

Docket No: CRP-074  
(2054/46)

MORPHOGEN TREATMENT OF GASTROINTESTINAL ULCERS

Reference to Related Applications

5 This application is a continuation-in-part of (1)  
USSN No. 07/752,764, filed August 30, 1991, which is a  
continuation-in-part of USSN 07/667,274, filed  
March 11, 1991; and (2) [CRP059CP], filed  
August 28, 1992, which is a continuation-in-part of  
[CRP068], filed August 28, 1992, and USSN 753,059,  
10 filed August 30, 1991, which is a continuation-in-part  
of USSN 667,274.

Field of the Invention

15 The invention relates generally to the treatment of  
gastrointestinal (GI) disorders. In particular, the  
invention relates to the treatment of ulcerative  
diseases within the gastrointestinal tract of a mammal.

Background of the Invention

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The luminal lining of the mammalian  
gastrointestinal tract (GI tract), which extends from  
the mouth cavity to the rectum, includes a protective  
layer of continually proliferating basal epithelial  
25 cells overlying a mucosal layer. Together, the basal  
epithelium and mucosa create the protective  
"gastrointestinal barrier." Disruption of this barrier  
results in lesions that can become infected and/or  
expose underlying tissue to the corrosive effect of

gastric juices. Gastrointestinal ulcerations can cause oral mucositis, gastric ulcers, necrotizing enterocolitis, regional ileitis, ulcerative colitis, regional enteritis (Crohn's disease), proctitis, and  
5 other forms of inflammatory bowel disease (IBD).

Ulcerative oral mucositis is a serious and dose-limiting toxic side effect of many forms of cancer therapies, including chemotherapy and radiation  
10 therapy. Oral mucositis accounts for significant pain and discomfort for these patients, and ranges in severity from redness and swelling to frank ulcerative lesions. Chemotherapeutic agents and radiation can kill or damage the epithelial cells lining the oral  
15 cavity. Such damage includes the inhibitory effect that chemotherapeutic agents may have on mitoses of the rapidly dividing cells of the oral basal epithelium. The severity of damage is related to the type and dose of chemotherapeutic agent(s) and concomitant therapy  
20 such as radiotherapy. Further, ulceration is hastened if sources of chronic irritation such as defective dental restorations, fractured teeth or ill-fitting dental prostheses are present. Oral mucositis most often affects the nonkeratinized mucosa of the cheeks,  
25 lips, soft palate, ventral surface of the tongue and floor of the mouth, approximately one to two weeks after cancer therapy. The lesions often become secondarily infected and become much harder to heal. The disruption in the oral mucosa results in a systemic  
30 portal of entry for the numerous microorganisms found in the mouth. Consequently, the oral cavity is the most frequently identifiable source of sepsis in the granulocytopenic cancer patient. Of primary concern are those patients undergoing: chemotherapy for cancer  
35 such as leukemia, breast cancer or as an adjuvant to

tumor removal; radiotherapy for head and neck cancer; and combined chemotherapy and radiotherapy for bone marrow transplants.

5        One source of oral mucositis can result from  
xerostomia, or chronic mouth dryness, which typically  
results from diminished or arrested salivary secretion  
or asialism. Salivary gland dysfunction or atrophy may  
10        result from tissue senescence in aged individuals, or  
from an organic disorder. Most frequently, xerostomia  
is an undesired side effect of a clinical or  
pharmaceutical therapy. Normally, saliva moistens the  
oral mucosal membrane, allowing for the dissolution and  
15        limited absorption of exogenous substances introduced  
into the oral cavity. In xerostomaic individuals  
irritating exogenous substances, including foods and  
medications, remain exposed to the mucosa and can cause  
inflammation and ulceration. A description of  
xerostomia-causing medications is described in  
20        Gallagher, et al. (1991) Current Opinion in Dentistry  
1:777-782.

Current therapy for mucositis is limited to either  
local or systemic palliation or topical antibacterial  
25        therapy. At present there is no effective treatment  
for mucositis. Therapy typically is limited to pain  
medications and treatment of secondary infection. In  
particular, recommendations have included treatment  
with topical anesthetics such as xylocaine, benzocaine  
30        and cocaine, treatment with solutions which coat the  
ulcerative lesions with a polysaccharide gel and use of  
antiseptic solutions such as Chlorhexadine. While all  
these treatments do provide some relief, none are  
directed to the actual healing of oral mucositis, which  
35        entails directly healing the mucosal epithelium cells.

Recently, certain local-acting growth factors, such as TGF- $\alpha$  have been shown to have some effect on ulcerative mucositis lesions at low concentrations, but less effect at higher concentrations (see US Pat.

5 No. 5,102870, issued April 7, 1992 to Florine et al.) The biphasic effect exhibited by such factors may limit their clinical utility. There remains a need for a therapy that inhibits ulcerative mucositis lesion formation and significantly enhances healing of lesions  
10 following their formation.

Gastrointestinal ulcer disease, in particular, peptic ulcers, affect 5-15% of the United States population. Peptic ulcers include gastric ulcers,  
15 which occur as lesions in the wall of the stomach, and duodenal ulcers, which are deep lesions that occur in the wall of the duodenum, i.e., the upper portion of the small intestine. Another ulcer disease, particularly worrisome to pediatricians, occurs in the  
20 premature infants. This condition, known as necrotizing enterocolitis, affects 10-15% of newborns having a birth weight of under 1.5 kg and results in severe ulceration of the small intestine, which frequently requires surgery. Gastric ulcers can result  
25 from an imbalance in factors which maintain the natural gastrointestinal barrier, including factors which neutralize corrosive gastric juices, such as the mucous bicarbonate, and other factors which protect the body from luminal damaging agents. Although current  
30 antiulcer therapeutics, including antisecretory products such as cimetidine and ranitidine, appear to be effective in healing duodenal ulcers, it is generally believed that they are effective because they reduce normal gastric acid secretion. While the  
35 reduction in acidity aids in the closure of the ulcer,

it also interferes with normal digestion. Accordingly, a high percentage of ulcers healed with current therapies recur within one year of therapy. The high rate of ulcer recurrence is thought to be at least partially attributable to the reduced number of mucus-producing cells in the scar tissue which is left at the site of the healed ulcer, rendering the area more vulnerable to rupture when the gastrointestinal acidity returns to normal.

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PCT Application No. PCT/US89/03467 discloses the use of an acid-resistant local-acting fibroblast growth factor to treat GI ulcers. US Pat. No. 5,043,329 discloses the use of phospholipids to treat ulcers of the gastrointestinal tract.

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Severe ulceration of the gastrointestinal mucosa also can spontaneously occur in the lower bowel (distal ileum and colon) in a spectrum of clinical disorders called inflammatory bowel disease (IBD). The two major diseases in this classification are ulcerative colitis and regional enteritis (Crohn's Disease) which are associated with severe mucosal ulceration (frequently penetrating the wall of the bowel and forming strictures and fistulas), severe mucosal and submucosal inflammation and edema, and fibrosis. Other forms of IBD include regional ileitis and proctitis. Clinically, patients with fulminant IBD can be severely ill with massive diarrhea, blood loss, dehydration, weight loss and fever. The prognosis of the disease is not good and frequently requires resection of the diseased tissue.

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It is an object of this invention to provide methods and compositions for maintaining the integrity of the gastrointestinal luminal lining in a mammal. Another object is to provide methods and compositions for regenerating basal epithelium and mucosa in ulcerated gastrointestinal tract barrier tissue, including the oral mucosa. Another object of the invention is to provide tissue protective methods and compositions that allow extension or enhancement of a chemical or radiotherapy. Another object is to provide methods and compositions capable of limiting the proliferation of epithelial cells, particularly the basal epithelial cells of the gastrointestinal tract. Still another object is to provide methods and compositions for substantially inhibiting inflammation normally associated with ulcerative diseases. Another object is to provide methods and compositions for protecting mucosal tissue from the tissue destructive effects associated with xerostomia. Yet another object is to provide methods and compositions for the treatment of oral mucositis, peptic ulcers, ulcerative colitis, regional enteritis, necrotizing enterocolitis, proctitis and other ulcerative diseases of the gastrointestinal tract.

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These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

Summary of the Invention

It now has been discovered that morphogenic proteins ("morphogen"), as defined herein, are useful as therapeutic methods and compositions for protecting the luminal lining of the gastrointestinal tract from ulceration, particularly in individuals at risk for ulcer formation. Specifically, the morphogens described herein can limit the proliferation of epithelial cells, inhibit the inflammation normally associated with ulcerative disease, inhibit scar tissue formation, and induce repair and regeneration of the ulcerated tissue.

In one aspect, the invention features compositions and therapeutic treatment methods that comprise the step of administering to a mammal a therapeutically effective amount of a morphogenic protein ("morphogen"), as defined herein, upon injury to all or a portion of the GI tract luminal lining, or in anticipation of such injury, for a time and at a concentration sufficient to maintain the integrity of the GI tract luminal lining, including repairing ulcerated tissue, and/or inhibiting damage thereto.

In another aspect, the invention features compositions and therapeutic treatment methods for maintaining the integrity of the GI tract luminal lining in a mammal which include administering to the mammal, upon injury to all or a portion of the GI tract luminal lining, or in anticipation of such injury, a compound that stimulates in vivo a therapeutically effective concentration of an endogenous morphogen within the body of the mammal sufficient to maintain the integrity of the luminal lining, including regenerating ulcerated tissue and/or inhibiting damage

thereto. These compounds are referred to herein as morphogen-stimulating agents, and are understood to include substances which, when administered to a mammal, act on cells in tissue(s) or organ(s) that normally are responsible for, or capable of, producing a morphogen and/or secreting a morphogen, and which cause the endogenous level of the morphogen to be altered. The agent may act, for example, by stimulating expression and/or secretion of an endogenous morphogen.

As used herein, "gastrointestinal tract" means the entire gastrointestinal tract of a mammal, from the mouth to the rectum, inclusive, including the mouth cavity, esophagus, stomach, upper and lower intestines, and colon. As used herein, "ulcer" refers to an open lesion or break of the integrity of the epithelial lining of the gastrointestinal tract, resulting in erosion of the underlying mucosa. "Maintaining the integrity of the luminal lining" means providing an effective morphogen concentration to the cells of the gastrointestinal tract luminal lining, the concentration being sufficient to substantially inhibit lesion formation in the basal epithelium of the gastrointestinal barrier, including stimulating the regeneration of damaged tissue and/or inhibiting additional damage thereto. "Protecting" mucosal tissue means providing a therapeutically effective morphogen concentration to the cells of the gastrointestinal tract luminal lining sufficient to inhibit the tissue damage associated with tissue ulceration, including stimulating regeneration of damaged tissue and/or inhibiting additional damage thereto. "Symptom-alleviating cofactor" refers to one or more pharmaceuticals which may be administered together with



the therapeutic agents of this invention and which alleviate or mitigate one or more of the symptoms typically associated with periodontal tissue loss. Exemplary cofactors include antibiotics, antiseptics, anti-viral and anti-fungal agents, non-steroidal antiinflammatory agents, anesthetics and analgesics, and antisecretory agents.

In preferred embodiments of the invention, the mammal is a human and ulcers treatable according to the invention include those found in the ileum which cause regional ileitis, those found in the colon which cause ulcerative colitis, regional enteritis (Crohn's disease), proctitis and other forms of inflammatory bowel disease (IBD), gastric ulcers such as those found in the stomach, small intestines, duodenum and esophagus; and ulcers found in the mouth. The compositions and methods described herein are particularly useful in treating mucositis lesions caused by chemotherapy or radiation therapy.

Because the morphogens described herein inhibit ulceration of the oral mucosa that typically results from cancer therapies, in another aspect, the invention provides cancer treatment methods and compositions that significantly reduce or inhibit the onset of oral mucositis in a patient. In addition, the morphogens described herein may be used in conjunction with existing chemical or radiation therapies to enhance their efficacy. Cancer chemical and radiation therapies currently in use often are limited in dose or duration by the onset of severe oral mucositis and/or the sepsis which often follows lesion formation. The morphogens described herein can inhibit lesion formation and, accordingly, their administration to a

patient as part of a cancer therapy may allow significant enhancement of current therapy doses and/or treatment times.

5       The morphogens described herein can limit cell proliferation in a proliferating epithelial cell population, thereby protecting these cells from the cytotoxic effects of chemotherapeutic and radiotherapeutic treatments. Accordingly, in another  
10   aspect, the invention provides methods and compositions for limiting the mitogenic activity of epithelial cells. This activity of the morphogens also has application for other diseases associated with proliferating epithelial cells, including psoriasis and  
15   other such skin tissue disorders. In addition, this activity of morphogens also may be useful to limit hair loss typically associated with cancer therapies.

20       The morphogens described also herein inhibit inflammation. Accordingly, in another aspect, the invention provides methods and compositions for inhibiting the inflammation associated with ulcerative disease.

25       The morphogens described herein also stimulate tissue morphogenesis at a site of tissue damage, inhibiting scar tissue formation at a lesion site. Accordingly, another aspect of the invention includes methods and compositions for inhibiting scar tissue  
30   formation at a lesion site.

In another aspect of the invention, the morphogens described herein are useful in protecting the mucosal membrane from the tissue destructive effects associated with xerostomia. The xerostomaic condition may be  
5 induced by a clinical therapy, including a cancer therapy, medication, diet or result from tissue senescence or an organic disorder of the salivary glands.

10 In one preferred embodiment, the morphogen or morphogen-stimulating agent is administered directly to the individual by topical administration, e.g., by coating the desired surface to be treated with the morphogen or morphogen-stimulating agent. For example,  
15 the therapeutic agent may be provided to the desired site by consuming a formulation containing the therapeutic agent in association with a compound capable of coating or adhering to the luminal lining surface. Such compounds include pectin-containing or  
20 sucralfate solutions such as are used in Milk of Magnesia and Kaopectate. For oral mucositis treatments, the agent may be provided in an oral rinse similar to a mouth wash that is swished around the mouth to coat the affected tissue, or disposed in a slow-dissolving  
25 lozenge or troche. Alternatively, the therapeutic agent may be provided to the site by physically applying or painting a formulation containing the morphogen or morphogen-stimulating agent to the site. Compositions for topical administration also may  
30 include a liquid adhesive to adhere the morphogen or morphogen-stimulating agent to the tissue surface. Useful adhesives include Zilactin, as is used in Orabase, hydroxypropylcellulose, and fibrinogen/thrombin solutions. Another potentially  
35 useful adhesive is the bioadhesive described in

compending USSN 627,323, the disclosure of which is incorporated herein by reference. The liquid adhesive may be painted onto the tissue surface, or formulated into an aerosol that is sprayed onto the affected  
5 tissue. For treatment of the lower bowel, the therapeutic agent also may be provided rectally, e.g., by suppository, foam, liquid ointment or cream, particularly for the treatment of ulcerations of the ileum and colon. In another embodiment of the  
10 invention, the morphogen or morphogen-stimulating agent is provided systemically, e.g., by parenteral administration.

In any treatment method of the invention,  
15 "administration of morphogen" refers to the administration of the morphogen, either alone or in combination with other molecules. For example, the mature form of the morphogen may be provided in association with its precursor "pro" domain, which is  
20 known to enhance the solubility of the protein in physiological solutions. Other useful molecules known to enhance protein solubility include casein and other milk components, as well as various serum proteins. Additional useful molecules which may be associated  
25 with the morphogen or morphogen-stimulating agent include tissue targeting molecules capable of directing the morphogen or morphogen-stimulating agent to epithelial mucosa tissue. Tissue targeting molecules envisioned to be useful in the treatment protocols of  
30 this invention include antibodies, antibody fragments or other binding proteins which interact specifically with surface molecules on GI barrier tissue cells. Non-steroidal anti-inflammatory agents which typically are targeted to inflamed tissue also may be used.

Still another useful tissue targeting molecule may comprise part or all of the morphogen precursor "pro" domain. Under naturally occurring conditions, the endogenous morphogens described herein may be synthesized in other tissues and transported to target tissue after secretion from the synthesizing tissue. For example, while the protein has been shown to be active in bone tissue, the primary source of OP-1 synthesis appears to be the tissue of the urogenic system (e.g., renal and bladder tissue), with secondary expression levels occurring in the brain, heart and lungs (see below.) Moreover, the protein has been identified in serum, saliva and various milk forms. In addition, the secreted form of the protein comprises the mature dimer in association with the pro domain of the intact morphogen sequence. Accordingly, the associated morphogen pro domains may act to target specific morphogens to different tissues in vivo.

Associated tissue targeting or solubility-enhancing molecules also may be covalently linked to the morphogen using standard chemical means, including acid-labile linkages, which likely will be preferentially cleaved in the acidic environment of the GI tract.

Finally, the morphogens or morphogen-stimulating agents provided herein also may be administered in combination with other molecules ("cofactors"), known to be beneficial in ulcer treatments, particularly cofactors capable of mitigating or alleviating symptoms typically associated with ulcerated tissue damage and/or loss. Examples of such cofactors include, analgesics/anesthetics such as xylocaine, and benzocaine; antiseptics such as chlorohexidine; anti-

bacterial, anti-viral and anti-fungal agents, including aminoglycosides, macrolides, penicillins, and cephalosporins; and antacids or antisecretory agents such as cimetidine or ramitidine.

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Among the morphogens useful in this invention are proteins originally identified as osteogenic proteins, such as the OP-1, OP-2 and CBMP2 proteins, as well as amino acid sequence-related proteins such as DPP (from Drosophila), Vgl (from Xenopus), Vgr-1 (from mouse, see U.S. 5,011,691 to Oppermann et al.), GDF-1 (from mouse, see Lee (1991) PNAS 88:4250-4254), all of which are presented in Table II and Seq. ID Nos.5-14), and the recently identified 60A protein (from Drosophila, Seq. ID No. 24, see Wharton et al. (1991) PNAS 88:9214-9218.) The members of this family, which include members of the TGF- $\beta$  super-family of proteins, share substantial amino acid sequence homology in their C-terminal regions. The proteins are translated as a precursor, having an N-terminal signal peptide sequence, typically less than about 30 residues, followed by a "pro" domain that is cleaved to yield the mature sequence. The "pro" form of the protein includes the pro domain and the mature domain, and forms a soluble species that appears to be the primary form secreted from cultured mammalian cells. The signal peptide is cleaved rapidly upon translation, at a cleavage site that can be predicted in a given sequence using the method of Von Heijne ((1986) Nucleic Acids Research 14:4683-4691.) Table I, below, describes the various morphogens identified to date, including their nomenclature as used herein, their Seq. ID references, and publication sources for the amino acid sequences for the full length proteins not included in the Seq. Listing. The disclosure of these publications is incorporated herein by reference.

TABLE I

5	"OP-1"	Refers generically to the group of morphogenically active proteins expressed from part or all of a DNA sequence encoding OP-1 protein, including allelic and species variants thereof, e.g., human OP-1 ("hOP-1", Seq. ID No. 5, mature protein amino acid sequence), or mouse OP-1 ("mOP-1", Seq. ID No. 6, mature protein amino acid sequence.) The conserved seven cysteine skeleton is defined by residues 38 to 139 of Seq. ID Nos. 5 and 6. The cDNA sequences and the amino acids encoding the full length proteins are provided in Seq. ID Nos. 16 and 17 (hOP1) and Seq. ID Nos. 18 and 19 (mOP1.) The mature proteins are defined by residues 293-431 (hOP1) and 292-430 (mOP1). The "pro" regions of the proteins, cleaved to yield the mature, morphogenically active proteins are defined essentially by residues 30-292 (hOP1) and residues 30-291 (mOP1).
10		
15		
20		
25		
	"OP-2"	refers generically to the group of active proteins expressed from part or all of a DNA sequence encoding OP-2 protein, including allelic and species variants thereof, e.g., human OP-2 ("hOP-2", Seq. ID No. 7, mature protein amino acid sequence) or mouse OP-2 ("mOP-2", Seq. ID No. 8, mature protein amino acid sequence). The conserved seven cysteine
30		
35		

5 skeleton is defined by residues 38 to 139  
of Seq. ID Nos. 7 and 8. The cDNA  
sequences and the amino acids encoding the  
full length proteins are provided in Seq.  
ID Nos. 20 and 21 (hOP2) and Seq. ID Nos.  
22 and 23 (mOP2.) The mature proteins are  
defined essentially by residues 264-402  
(hOP2) and 261-399 (mOP2). The "pro"  
regions of the proteins, cleaved to yield  
10 the mature, morphogenically active  
proteins likely are defined essentially by  
residues 18-263 (hOP2) and residues 18-260  
(mOP2). (Another cleavage site also  
occurs 21 residues upstream for hOP-2  
15 protein.)

"CBMP2" refers generically to the morphogenically  
active proteins expressed from a DNA  
sequence encoding the CBMP2 proteins,  
20 including allelic and species variants  
thereof, e.g., human CBMP2A ("CBMP2A(fx)",  
Seq ID No. 9) or human CBMP2B DNA  
("CBMP2B(fx)", Seq. ID No. 10). The amino  
acid sequence for the full length  
25 proteins, referred to in the literature as  
BMP2A and BMP2B, or BMP2 and BMP4, appear  
in Wozney, et al. (1988) Science  
242:1528-1534. The pro domain for BMP2  
(BMP2A) likely includes residues 25-248 or  
30 25-282; the mature protein, residues  
249-396 or 283-396. The pro domain for  
BMP4 (BMP2B) likely includes residues 25-  
256 or 25-292; the mature protein,  
residues 257-408 or 293-408.

35



- 5 "DPP(fx)" refers to protein sequences encoded by the  
Drosophila DPP gene and defining the  
conserved seven cysteine skeleton (Seq. ID  
No. 11). The amino acid sequence for the  
full length protein appears in Padgett, et  
al (1987) Nature 325: 81-84. The pro  
domain likely extends from the signal  
peptide cleavage site to residue 456; the  
mature protein likely is defined by  
residues 457-588.
- 15 "Vgl(fx)" refers to protein sequences encoded by the  
Xenopus Vgl gene and defining the  
conserved seven cysteine skeleton (Seq. ID  
No. 12). The amino acid sequence for the  
full length protein appears in  
Weeks (1987) Cell 51: 861-867. The pro  
domain likely extends from the signal  
peptide cleavage site to residue 246; the  
mature protein likely is defined by  
residues 247-360.
- 25 "Vgr-1(fx)" refers to protein sequences encoded by the  
murine Vgr-1 gene and defining the  
conserved seven cysteine skeleton (Seq. ID  
No. 13). The amino acid sequence for the  
full length protein appears in Lyons, et  
al, (1989) PNAS 86: 4554-4558. The pro  
domain likely extends from the signal  
peptide cleavage site to residue 299; the  
mature protein likely is defined by  
residues 300-438.

"GDF-1(fx)" refers to protein sequences encoded by the human GDF-1 gene and defining the conserved seven cysteine skeleton (Seq. ID No. 14). The cDNA and encoded amino sequence for the full length protein is provided in Seq. ID. No. 32. The pro domain likely extends from the signal peptide cleavage site to residue 214; the mature protein likely is defined by residues 215-372.

"60A" refers generically to the morphogenically active proteins expressed from part or all of a DNA sequence (from the Drosophila 60A gene) encoding the 60A proteins (see Seq. ID No. 24 wherein the cDNA and encoded amino acid sequence for the full length protein is provided). "60A(fx)" refers to the protein sequences defining the conserved seven cysteine skeleton (residues 354 to 455 of Seq. ID No. 24.) The pro domain likely extends from the signal peptide cleavage site to residue 324; the mature protein likely is defined by residues 325-455.

"BMP3(fx)" refers to protein sequences encoded by the human BMP3 gene and defining the conserved seven cysteine skeleton (Seq. ID No. 26). The amino acid sequence for the full length protein appears in Wozney et al. (1988) Science 242: 1528-1534. The pro domain likely extends from the signal peptide cleavage site to residue 290; the mature protein likely is defined by residues 291-472.

"BMP5(fx)" refers to protein sequences encoded by the human BMP5 gene and defining the conserved seven cysteine skeleton (Seq. ID No. 27).  
5 The amino acid sequence for the full length protein appears in Celeste, et al. (1991) PNAS 87: 9843-9847. The pro domain likely extends from the signal peptide cleavage site to residue 316; the mature protein likely is defined by residues  
10 317-454.

"BMP6(fx)" refers to protein sequences encoded by the human BMP6 gene and defining the conserved seven cysteine skeleton (Seq. ID No. 28).  
15 The amino acid sequence for the full length protein appears in Celeste, et al. (1990) PNAS 87: 9843-5847. The pro domain likely extends from the signal peptide  
20 cleavage site to residue 374; the mature sequence likely includes residues 375-513.

The OP-2 proteins have an additional cysteine residue in this region (e.g., see residue 41 of Seq. ID  
25 Nos. 7 and 8), in addition to the conserved cysteine skeleton in common with the other proteins in this family. The GDF-1 protein has a four amino acid insert within the conserved skeleton (residues 44-47 of Seq. ID No. 14) but this insert likely does not interfere  
30 with the relationship of the cysteines in the folded structure. In addition, the CBMP2 proteins are missing one amino acid residue within the cysteine skeleton.

The morphogens are inactive when reduced, but are active as oxidized homodimers and when oxidized in combination with other morphogens of this invention. Thus, as defined herein, a morphogen is a dimeric protein comprising a pair of polypeptide chains, wherein each polypeptide chain comprises at least the C-terminal six cysteine skeleton defined by residues 43-139 of Seq. ID No. 5, including functionally equivalent arrangements of these cysteines (e.g., amino acid insertions or deletions which alter the linear arrangement of the cysteines in the sequence but not their relationship in the folded structure), such that, when the polypeptide chains are folded, the dimeric protein species comprising the pair of polypeptide chains has the appropriate three-dimensional structure, including the appropriate intra- or inter-chain disulfide bonds such that the protein is capable of acting as a morphogen as defined herein. Specifically, the morphogens generally are capable of all of the following biological functions in a morphogenically permissive environment: stimulating proliferation of progenitor cells; stimulating the differentiation of progenitor cells; stimulating the proliferation of differentiated cells; and supporting the growth and maintenance of differentiated cells, including the "redifferentiation" of transformed cells. In addition, it is also anticipated that these morphogens are capable of inducing redifferentiation of committed cells under appropriate environmental conditions.

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In one preferred aspect, the morphogens of this invention comprise one of two species of generic amino acid sequences: Generic Sequence 1 (Seq. ID No. 1) or Generic Sequence 2 (Seq. ID No. 2); where each Xaa indicates one of the 20 naturally-occurring L-isomer,

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$\alpha$ -amino acids or a derivative thereof. Generic Sequence 1 comprises the conserved six cysteine skeleton and Generic Sequence 2 comprises the conserved six cysteine skeleton plus the additional cysteine identified in OP-2 (see residue 36, Seq. ID No. 2). In another preferred aspect, these sequences further comprise the following additional sequence at their N-terminus:

10                   Cys Xaa Xaa Xaa Xaa (Seq. ID No. 15)  
                    1                                   5

Preferred amino acid sequences within the foregoing generic sequences include: Generic Sequence 3 (Seq. ID No. 3), Generic Sequence 4 (Seq. ID No. 4), Generic Sequence 5 (Seq. ID No. 30) and Generic Sequence 6 (Seq. ID No. 31), listed below. These Generic Sequences accommodate the homologies shared among the various preferred members of this morphogen family identified in Table II, as well as the amino acid sequence variation among them. Specifically, Generic Sequences 3 and 4 are composite amino acid sequences of the following proteins presented in Table II and identified in Seq. ID Nos. 5-14: human OP-1 (hOP-1, Seq. ID Nos. 5 and 16-17), mouse OP-1 (mOP-1, Seq. ID Nos. 6 and 18-19), human and mouse OP-2 (Seq. ID Nos. 7, 8, and 20-22), CBMP2A (Seq. ID No. 9), CBMP2B (Seq. ID No. 10), DPP (from Drosophila, Seq. ID No. 11), Vgl, (from Xenopus, Seq. ID No. 12), Vgr-1 (from mouse, Seq. ID No. 13), and GDF-1 (from mouse, Seq. ID No. 14.) The generic sequences include both the amino acid identity shared by the sequences in Table II, as well as alternative residues for the variable positions within the sequence. Note that these generic sequences allow for an additional

cysteine at position 41 or 46 in Generic Sequences 3 or 4, respectively, providing an appropriate cysteine skeleton where inter- or intramolecular disulfide bonds can form, and contain certain critical amino acids which influence the tertiary structure of the proteins.

Generic Sequence 3

		Leu	Tyr	Val	Xaa	Phe		
10		1			5			
		Xaa	Xaa	Xaa	Gly	Trp	Xaa	Xaa
					10			
		Xaa	Ala	Pro	Xaa	Gly	Xaa	Xaa
		15				20		
15		Xaa	Tyr	Cys	Xaa	Gly	Xaa	Cys
			25				30	
		Xaa	Pro	Xaa	Xaa	Xaa	Xaa	Xaa
					35			
		Xaa	Xaa	Xaa	Asn	His	Ala	Xaa
20			40				45	
		Xaa	Xaa	Leu	Xaa	Xaa	Xaa	Xaa
					50			
		Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Cys
		55					60	
25		Cys	Xaa	Pro	Xaa	Xaa	Xaa	Xaa
					65			
		Xaa	Xaa	Xaa	Leu	Xaa	Xaa	Xaa
		70					75	
		Xaa	Xaa	Xaa	Xaa	Val	Xaa	Leu
30					80			
	A	Xaa	Xaa	Xaa	Xaa	Met	Xaa	Val
		85					90	
		Xaa	Cys	Gly	Cys	Xaa		
					95			
35								

wherein each Xaa is independently selected from a group of one or more specified amino acids defined as follows: "Res." means "residue" and Xaa at res.4 = (Ser, Asp or Glu); Xaa at res.6 = (Arg, Gln, Ser or Lys); Xaa at res.7 = (Asp or Glu); Xaa at res.8 = (Leu or Val); Xaa at res.11 = (Gln, Leu, Asp, His or Asn); Xaa at res.12 = (Asp, Arg or Asn); Xaa at res.14 = (Ile or Val); Xaa at res.15 = (Ile or Val); Xaa at res.18 = (Glu, Gln, Leu, Lys, Pro or Arg); Xaa at res.20 = (Tyr or Phe); Xaa at res.21 = (Ala, Ser, Asp, Met, His, Leu or Gln); Xaa at res.23 = (Tyr, Asn or Phe); Xaa at res.26 = (Glu, His, Tyr, Asp or Gln); Xaa at res.28 = (Glu, Lys, Asp or Gln); Xaa at res.30 = (Ala, Ser, Pro or Gln); Xaa at res.31 = (Phe, Leu or Tyr); Xaa at res.33 = (Leu or Val); Xaa at res.34 = (Asn, Asp, Ala or Thr); Xaa at res.35 = (Ser, Asp, Glu, Leu or Ala); Xaa at res.36 = (Tyr, Cys, His, Ser or Ile); Xaa at res.37 = (Met, Phe, Gly or Leu); Xaa at res.38 = (Asn or Ser); Xaa at res.39 = (Ala, Ser or Gly); Xaa at res.40 = (Thr, Leu or Ser); Xaa at res.44 = (Ile or Val); Xaa at res.45 = (Val or Leu); Xaa at res.46 = (Gln or Arg); Xaa at res.47 = (Thr, Ala or Ser); Xaa at res.49 = (Val or Met); Xaa at res.50 = (His or Asn); Xaa at res.51 = (Phe, Leu, Asn, Ser, Ala or Val); Xaa at res.52 = (Ile, Met, Asn, Ala or Val); Xaa at res.53 = (Asn, Lys, Ala or Glu); Xaa at res.54 = (Pro or Ser); Xaa at res.55 = (Glu, Asp, Asn, or Gly); Xaa at res.56 = (Thr, Ala, Val, Lys, Asp, Tyr, Ser or Ala); Xaa at res.57 = (Val, Ala or Ile); Xaa at res.58 = (Pro or Asp); Xaa at res.59 = (Lys or Leu); Xaa at res.60 = (Pro or Ala); Xaa at res.63 = (Ala or Val); Xaa at res.65 = (Thr or Ala); Xaa at res.66 = (Gln, Lys, Arg or Glu); Xaa at res.67 = (Leu, Met or Val); Xaa at res.68 = (Asn, Ser or Asp); Xaa at res.69 = (Ala, Pro or Ser); Xaa at res.70 = (Ile, Thr or Val); Xaa at

res.71 = (Ser or Ala); Xaa at res.72 = (Val or Met);  
 Xaa at res.74 = (Tyr or Phe); Xaa at res.75 = (Phe, Tyr  
 or Leu); Xaa at res.76 = (Asp or Asn); Xaa at res.77 =  
 (Asp, Glu, Asn or Ser); Xaa at res.78 = (Ser, Gln, Asn  
 5 or Tyr); Xaa at res.79 = (Ser, Asn, Asp or Glu); Xaa at  
 res.80 = (Asn, Thr or Lys); Xaa at res.82 = (Ile or  
 Val); Xaa at res.84 = (Lys or Arg); Xaa at res.85 =  
 (Lys, Asn, Gln or His); Xaa at res.86 = (Tyr or His);  
 Xaa at res.87 = (Arg, Gln or Glu); Xaa at res.88 =  
 10 (Asn, Glu or Asp); Xaa at res.90 = (Val, Thr or Ala);  
 Xaa at res.92 = (Arg, Lys, Val, Asp or Glu); Xaa at  
 res.93 = (Ala, Gly or Glu); and Xaa at res.97 = (His or  
 Arg);

15 Generic Sequence 4

	Cys	Xaa	Xaa	Xaa	Xaa	Leu	Tyr	Val	Xaa	Phe
	1				5					10
	Xaa	Xaa	Xaa	Gly	Trp	Xaa	Xaa	Trp	Xaa	
20					15					
	Xaa	Ala	Pro	Xaa	Gly	Xaa	Xaa	Ala		
	20				25					
	Xaa	Tyr	Cys	Xaa	Gly	Xaa	Cys	Xaa		
			30					35		
25	Xaa	Pro	Xaa	Xaa	Xaa	Xaa	Xaa			
					40					
	Xaa	Xaa	Xaa	Asn	His	Ala	Xaa	Xaa		
				45				50		
	Xaa	Xaa	Leu	Xaa	Xaa	Xaa	Xaa	Xaa		
30					55					
	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Cys		
	60							65		
	Cys	Xaa	Pro	Xaa	Xaa	Xaa	Xaa	Xaa		
					70					



Xaa Xaa Xaa Leu Xaa Xaa Xaa  
 75 80  
 Xaa Xaa Xaa Xaa Val Xaa Leu Xaa  
 85  
 5 Xaa Xaa Xaa Xaa Met Xaa Val Xaa  
 90 95  
 Xaa Cys Gly Cys Xaa  
 100

- 10 wherein each Xaa is independently selected from a group  
 of one or more specified amino acids as defined by the  
 following: "Res." means "residue" and Xaa at res.2 =  
 (Lys or Arg); Xaa at res.3 = (Lys or Arg); Xaa at  
 res.4 = (His or Arg); Xaa at res.5 = (Glu, Ser, His,  
 15 Gly, Arg or Pro); Xaa at res.9 = (Ser, Asp or Glu); Xaa  
 at res.11 = (Arg, Gln, Ser or Lys); Xaa at res.12 =  
 (Asp or Glu); Xaa at res.13 = (Leu or Val); Xaa at  
 res.16 = (Gln, Leu, Asp, His or Asn); Xaa at res.17 =  
 (Asp, Arg, or Asn); Xaa at res.19 = (Ile or Val); Xaa  
 20 at res.20 = (Ile or Val); Xaa at res.23 = (Glu, Gln,  
 Leu, Lys, Pro or Arg); Xaa at res.25 = (Tyr or Phe);  
 Xaa at res.26 = (Ala, Ser, Asp, Met, His, Leu, or Gln);  
 Xaa at res.28 = (Tyr, Asn or Phe); Xaa at res.31 =  
 (Glu, His, Tyr, Asp or Gln); Xaa at res.33 = Glu, Lys,  
 25 Asp or Gln); Xaa at res.35 = (Ala, Ser or Pro); Xaa at  
 res.36 = (Phe, Leu or Tyr); Xaa at res.38 = (Leu or  
 Val); Xaa at res.39 = (Asn, Asp, Ala or Thr); Xaa at  
 res.40 = (Ser, Asp, Glu, Leu or Ala); Xaa at res.41 =  
 (Tyr, Cys, His, Ser or Ile); Xaa at res.42 = (Met, Phe,  
 30 Gly or Leu); Xaa at res.44 = (Ala, Ser or Gly); Xaa at  
 res.45 = (Thr, Leu or Ser); Xaa at res.49 = (Ile or  
 Val); Xaa at res.50 = (Val or Leu); Xaa at res.51 =  
 (Gln or Arg); Xaa at res.52 = (Thr, Ala or Ser); Xaa at  
 res.54 = (Val or Met); Xaa at res.55 = (His or Asn);  
 35 Xaa at res.56 = (Phe, Leu, Asn, Ser, Ala or Val); Xaa

at res.57 = (Ile, Met, Asn, Ala or Val); Xaa at  
res.58 = (Asn, Lys, Ala or Glu); Xaa at res.59 = (Pro  
or Ser); Xaa at res.60 = (Glu, Asp, or Gly); Xaa at  
res.61 = (Thr, Ala, Val, Lys, Asp, Tyr, Ser or Ala);  
5 Xaa at res.62 = (Val, Ala or Ile); Xaa at res.63 = (Pro  
or Asp); Xaa at res.64 = (Lys or Leu); Xaa at res.65 =  
(Pro or Ala); Xaa at res.68 = (Ala or Val); Xaa at  
res.70 = (Thr or Ala); Xaa at res.71 = (Gln, Lys, Arg  
or Glu); Xaa at res.72 = (Leu, Met or Val); Xaa at  
10 res.73 = (Asn, Ser or Asp); Xaa at res.74 = (Ala, Pro  
or Ser); Xaa at res.75 = (Ile, Thr or Val); Xaa at  
res.76 = (Ser or Ala); Xaa at res.77 = (Val or Met);  
Xaa at res.79 = (Tyr or Phe); Xaa at res.80 = (Phe, Tyr  
or Leu); Xaa at res.81 = (Asp or Asn); Xaa at res.82 =  
15 (Asp, Glu, Asn or Ser); Xaa at res.83 = (Ser, Gln, Asn  
or Tyr); Xaa at res.84 = (Ser, Asn, Asp or Glu); Xaa at  
res.85 = (Asn, Thr or Lys); Xaa at res.87 = (Ile or  
Val); Xaa at res.89 = (Lys or Arg); Xaa at res.90 =  
(Lys, Asn, Gln or His); Xaa at res.91 = (Tyr or His);  
20 Xaa at res.92 = (Arg, Gln or Glu); Xaa at res.93 =  
(Asn, Glu or Asp); Xaa at res.95 = (Val, Thr or Ala);  
Xaa at res.97 = (Arg, Lys, Val, Asp or Glu); Xaa at  
res.98 = (Ala, Gly or Glu); and Xaa at res.102 = (His  
or Arg).

25

Similarly, Generic Sequence 5 (Seq. ID No. 30) and  
Generic Sequence 6 (Seq. ID No. 31) accommodate the  
homologies shared among all the morphogen protein  
family members identified in Table II. Specifically,  
30 Generic Sequences 5 and 6 are composite amino acid  
sequences of human OP-1 (hOP-1, Seq. ID Nos. 5 and 16-  
17), mouse OP-1 (mOP-1, Seq. ID Nos. 6 and 18-19),  
human and mouse OP-2 (Seq. ID Nos. 7, 8, and 20-22),  
CBMP2A (Seq. ID No. 9), CBMP2B (Seq. ID No. 10), DPP  
35 (from Drosophila, Seq. ID No. 11), Vgl, (from Xenopus,

Seq. ID No. 12), Vgr-1 (from mouse, Seq. ID No. 13),  
and GDF-1 (from mouse, Seq. ID No. 14), human BMP3  
(Seq. ID No. 26), human BMP5 (Seq. ID No. 27), human  
BMP6 (Seq. ID No. 28) and 60(A) (from Drosophila, Seq.  
5 ID Nos. 24-25). The generic sequences include both the  
amino acid identity shared by these sequences in the  
C-terminal domain, defined by the six and seven  
cysteine skeletons (Generic Sequences 5 and 6,  
respectively), as well as alternative residues for the  
10 variable positions within the sequence. As for Generic  
Sequences 3 and 4, Generic Sequences 5 and 6 allow for  
an additional cysteine at position 41 (Generic Sequence  
5) or position 46 (Generic Sequence 6), providing an  
appropriate cysteine skeleton where inter- or  
15 intramolecular disulfide bonds can form, and containing  
certain critical amino acids which influence the  
tertiary structure of the proteins.

Generic Sequence 5

20  
Leu Xaa Xaa Xaa Phe  
1 5  
Xaa Xaa Xaa Gly Trp Xaa Xaa Trp Xaa  
10  
25 Xaa Xaa Pro Xaa Xaa Xaa Xaa Ala  
15 20  
Xaa Tyr Cys Xaa Gly Xaa Cys Xaa  
25 30  
Xaa Pro Xaa Xaa Xaa Xaa Xaa  
30 35  
Xaa Xaa Xaa Asn His Ala Xaa Xaa  
40 45  
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa  
50

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys  
           55                          60  
 Cys Xaa Pro Xaa Xaa Xaa Xaa Xaa  
                           65  
 5     Xaa Xaa Xaa Leu Xaa Xaa Xaa  
           70                          75  
       Xaa Xaa Xaa Xaa Val Xaa Leu Xaa  
                           80  
       Xaa Xaa Xaa Xaa Met Xaa Val Xaa  
 10     85                          90  
       Xaa Cys Xaa Cys Xaa  
                           95

wherein each Xaa is independently selected from a group  
 15 of one or more specified amino acids defined as  
 follows: "Res." means "residue" and Xaa at res.2 =  
 (Tyr or Lys); Xaa at res.3 = Val or Ile); Xaa at  
 res.4 = (Ser, Asp or Glu); Xaa at res.6 = (Arg, Gln,  
 Ser, Lys or Ala); Xaa at res.7 = (Asp, Glu or Lys); Xaa  
 20 at res.8 = (Leu, Val or Ile); Xaa at res.11 = (Gln,  
 Leu, Asp, His, Asn or Ser); Xaa at res.12 = (Asp, Arg,  
 Asn or Glu); Xaa at res.14 = (Ile or Val); Xaa at  
 res.15 = (Ile or Val); Xaa at res.16 (Ala or Ser); Xaa  
 at res.18 = (Glu, Gln, Leu, Lys, Pro or Arg); Xaa at  
 25 res.19 = (Gly or Ser); Xaa at res.20 = (Tyr or Phe);  
 Xaa at res.21 = (Ala, Ser, Asp, Met, His, Gln, Leu or  
 Gly); Xaa at res.23 = (Tyr, Asn or Phe); Xaa at  
 res.26 = (Glu, His, Tyr, Asp, Gln or Ser); Xaa at  
 res.28 = (Glu, Lys, Asp, Gln or Ala); Xaa at res.30 =  
 30 (Ala, Ser, Pro, Gln or Asn); Xaa at res.31 = (Phe, Leu  
 or Tyr); Xaa at res.33 = (Leu, Val or Met); Xaa at  
 res.34 = (Asn, Asp, Ala, Thr or Pro); Xaa at res.35 =  
 (Ser, Asp, Glu, Leu, Ala or Lys); Xaa at res.36 = (Tyr,  
 Cys, His, Ser or Ile); Xaa at res.37 = (Met, Phe, Gly  
 35 or Leu); Xaa at res.38 = (Asn, Ser or Lys); Xaa at

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res.39 = (Ala, Ser, Gly or Pro); Xaa at res.40 = (Thr, Leu or Ser); Xaa at res.44 = (Ile, Val or Thr); Xaa at res.45 = (Val, Leu or Ile); Xaa at res.46 = (Gln or Arg); Xaa at res.47 = (Thr, Ala or Ser); Xaa at  
5 res.48 = (Leu or Ile); Xaa at res.49 = (Val or Met); Xaa at res.50 = (His, Asn or Arg); Xaa at res.51 = (Phe, Leu, Asn, Ser, Ala or Val); Xaa at res.52 = (Ile, Met, Asn, Ala, Val or Leu); Xaa at res.53 = (Asn, Lys, Ala, Glu, Gly or Phe); Xaa at res.54 = (Pro, Ser or  
10 Val); Xaa at res.55 = (Glu, Asp, Asn, Gly, Val or Lys); Xaa at res.56 = (Thr, Ala, Val, Lys, Asp, Tyr, Ser, Ala, Pro or His); Xaa at res.57 = (Val, Ala or Ile); Xaa at res.58 = (Pro or Asp); Xaa at res.59 = (Lys, Leu or Glu); Xaa at res.60 = (Pro or Ala); Xaa at res.63 =  
15 (Ala or Val); Xaa at res.65 = (Thr, Ala or Glu); Xaa at res.66 = (Gln, Lys, Arg or Glu); Xaa at res.67 = (Leu, Met or Val); Xaa at res.68 = (Asn, Ser, Asp or Gly); Xaa at res.69 = (Ala, Pro or Ser); Xaa at res.70 = (Ile, Thr, Val or Leu); Xaa at res.71 = (Ser, Ala or  
20 Pro); Xaa at res.72 = (Val, Met or Ile); Xaa at res.74 = (Tyr or Phe); Xaa at res.75 = (Phe, Tyr, Leu or His); Xaa at res.76 = (Asp, Asn or Leu); Xaa at res.77 = (Asp, Glu, Asn or Ser); Xaa at res.78 = (Ser, Gln, Asn, Tyr or Asp); Xaa at res.79 = (Ser, Asn, Asp,  
25 Glu or Lys); Xaa at res.80 = (Asn, Thr or Lys); Xaa at res.82 = (Ile, Val or Asn); Xaa at res.84 = (Lys or Arg); Xaa at res.85 = (Lys, Asn, Gln, His or Val); Xaa at res.86 = (Tyr or His); Xaa at res.87 = (Arg, Gln, Glu or Pro); Xaa at res.88 = (Asn, Glu or Asp); Xaa at  
30 res.90 = (Val, Thr, Ala or Ile); Xaa at res.92 = (Arg, Lys, Val, Asp or Glu); Xaa at res.93 = (Ala, Gly, Glu or Ser); Xaa at res.95 = (Gly or Ala) and Xaa at res.97 = (His or Arg).

Generic Sequence 6

	Cys	Xaa	Xaa	Xaa	Xaa	Leu	Xaa	Xaa	Xaa	Phe
	1					5				10
5	Xaa	Xaa	Xaa	Gly	Trp	Xaa	Xaa	Trp	Xaa	
						15				
	Xaa	Xaa	Pro	Xaa	Xaa	Xaa	Xaa	Ala		
	20					25				
	Xaa	Tyr	Cys	Xaa	Gly	Xaa	Cys	Xaa		
10			30					35		
	Xaa	Pro	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa		
						40				
	Xaa	Xaa	Xaa	Asn	His	Ala	Xaa	Xaa		
						45		50		
15	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa		
						55				
	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Cys		
						60		65		
	Cys	Xaa	Pro	Xaa	Xaa	Xaa	Xaa	Xaa		
20						70				
	Xaa	Xaa	Xaa	Leu	Xaa	Xaa	Xaa			
						75		80		
	Xaa	Xaa	Xaa	Xaa	Val	Xaa	Leu	Xaa		
						85				
25	Xaa	Xaa	Xaa	Xaa	Met	Xaa	Val	Xaa		
						90		95		
	Xaa	Cys	Xaa	Cys	Xaa					
						100				

30 wherein each Xaa is independently selected from a group  
of one or more specified amino acids as defined by the  
following: "Res." means "residue" and Xaa at res.2 =  
(Lys, Arg, Ala or Gln); Xaa at res.3 = (Lys, Arg or  
Met); Xaa at res.4 = (His, Arg or Gln); Xaa at res.5 =  
35 (Glu, Ser, His, Gly, Arg, Pro, Thr, or Tyr); Xaa at

res.7 = (Tyr or Lys); Xaa at res.8 = (Val or Ile); Xaa  
at res.9 = (Ser, Asp or Glu); Xaa at res.11 = (Arg,  
Gln, Ser, Lys or Ala); Xaa at res.12 = (Asp, Glu, or  
Lys); Xaa at res.13 = (Leu, Val or Ile); Xaa at  
5 res.16 = (Gln, Leu, Asp, His, Asn or Ser); Xaa at  
res.17 = (Asp, Arg, Asn or Glu); Xaa at res.19 = (Ile  
or Val); Xaa at res.20 = (Ile or Val); Xaa at res.21 =  
(Ala or Ser); Xaa at res.23 = (Glu, Gln, Leu, Lys, Pro  
or Arg); Xaa at res.24 = (Gly or Ser); Xaa at res.25 =  
10 (Tyr or Phe); Xaa at res.26 = (Ala, Ser, Asp, Met, His,  
Gln, Leu, or Gly); Xaa at res.28 = (Tyr, Asn or Phe);  
Xaa at res.31 = (Glu, His, Tyr, Asp, Gln or Ser); Xaa  
at res.33 = Glu, Lys, Asp, Gln or Ala); Xaa at res.35 =  
(Ala, Ser, Pro, Gln or Asn); Xaa at res.36 = (Phe, Leu  
15 or Tyr); Xaa at res.38 = (Leu, Val or Met); Xaa at  
res.39 = (Asn, Asp, Ala, Thr or Pro); Xaa at res.40 =  
(Ser, Asp, Glu, Leu, Ala or Lys); Xaa at res.41 = (Tyr,  
Cys, His, Ser or Ile); Xaa at res.42 = (Met, Phe, Gly  
or Leu); Xaa at res.43 = (Asn, Ser or Lys); Xaa at  
20 res.44 = (Ala, Ser, Gly or Pro); Xaa at res.45 = (Thr,  
Leu or Ser); Xaa at res.49 = (Ile, Val or Thr); Xaa at  
res.50 = (Val, Leu or Ile); Xaa at res.51 = (Gln or  
Arg); Xaa at res.52 = (Thr, Ala or Ser); Xaa at  
res.53 = (Leu or Ile); Xaa at res.54 = (Val or Met);  
25 Xaa at res.55 = (His, Asn or Arg); Xaa at res.56 =  
(Phe, Leu, Asn, Ser, Ala or Val); Xaa at res.57 = (Ile,  
Met, Asn, Ala, Val or Leu); Xaa at res.58 = (Asn, Lys,  
Ala, Glu, Gly or Phe); Xaa at res.59 = (Pro, Ser or  
Val); Xaa at res.60 = (Glu, Asp, Gly, Val or Lys); Xaa  
30 at res.61 = (Thr, Ala, Val, Lys, Asp, Tyr, Ser, Ala,  
Pro or His); Xaa at res.62 = (Val, Ala or Ile); Xaa at  
res.63 = (Pro or Asp); Xaa at res.64 = (Lys, Leu or  
Glu); Xaa at res.65 = (Pro or Ala); Xaa at res.68 =  
(Ala or Val); Xaa at res.70 = (Thr, Ala or Glu); Xaa at  
35 res.71 = (Gln, Lys, Arg or Glu); Xaa at res.72 = (Leu,

Met or Val); Xaa at res.73 = (Asn, Ser, Asp or Gly);  
Xaa at res.74 = (Ala, Pro or Ser); Xaa at res.75 =  
(Ile, Thr, Val or Leu); Xaa at res.76 = (Ser, Ala or  
Pro); Xaa at res.77 = (Val, Met or Ile); Xaa at  
5 res.79 = (Tyr or Phe); Xaa at res.80 = (Phe, Tyr, Leu  
or His); Xaa at res.81 = (Asp, Asn or Leu); Xaa at  
res.82 = (Asp, Glu, Asn or Ser); Xaa at res.83 = (Ser,  
Gln, Asn, Tyr or Asp); Xaa at res.84 = (Ser, Asn, Asp,  
Glu or Lys); Xaa at res.85 = (Asn, Thr or Lys); Xaa at  
10 res.87 = (Ile, Val or Asn); Xaa at res.89 = (Lys or  
Arg); Xaa at res.90 = (Lys, Asn, Gln, His or Val); Xaa  
at res.91 = (Tyr or His); Xaa at res.92 = (Arg, Gln,  
Glu or Pro); Xaa at res.93 = (Asn, Glu or Asp); Xaa at  
res.95 = (Val, Thr, Ala or Ile); Xaa at res.97 = (Arg,  
15 Lys, Val, Asp or Glu); Xaa at res.98 = (Ala, Gly, Glu  
or Ser); Xaa at res.100 = (Gly or Ala); and Xaa at  
res.102 = (His or Arg).

Particularly useful sequences for use as morphogens  
20 in this invention include the C-terminal domains, e.g.,  
the C-terminal 96-102 amino acid residues of Vgl,  
Vgr-1, DPP, OP-1, OP-2, CBMP-2A, CBMP-2B, GDF-1 (see  
Table II, below, and Seq. ID Nos. 5-14), as well as  
proteins comprising the C-terminal domains of 60A,  
25 BMP3, BMP5 and BMP6 (see Seq. ID Nos. 24-28), all of  
which include at least the conserved six or seven  
cysteine skeleton. In addition, biosynthetic  
constructs designed from the generic sequences, such as  
COP-1, 3-5, 7, 16, disclosed in U.S. Pat. No.  
30 5,011,691, also are useful. Other sequences include  
the inhibins/activin proteins (see, for example, U.S.  
Pat. Nos. 4,968,590 and 5,011,691). Accordingly, other  
useful sequences are those sharing at least 70% amino  
acid sequence homology or "similarity", and preferably  
35 80% homology or similarity with any of the sequences



above. These are anticipated to include allelic and species variants and mutants, and biosynthetic muteins, as well as novel members of this morphogenic family of proteins. Particularly envisioned in the family of

5 related proteins are those proteins exhibiting morphogenic activity and wherein the amino acid changes from the preferred sequences include conservative changes, e.g., those as defined by Dayoff et al., Atlas of Protein Sequence and Structure; vol. 5, Suppl. 3,  
-10 pp. 345-362, (M.O. Dayoff, ed., Nat'l BioMed. Research Fdn., Washington, D.C. 1979). As used herein, potentially useful sequences are aligned with a known morphogen sequence using the method of Needleman et al. ((1970) J.Mol.Biol. 48:443-453) and identities  
15 calculated by the Align program (DNASTar, Inc.). "Homology" or "similarity" as used herein includes allowed conservative changes as defined by Dayoff et al.

20 The currently most preferred protein sequences useful as morphogens in this invention include those having greater than 60% identity, preferably greater than 65% identity, with the amino acid sequence defining the conserved six cysteine skeleton of hOP1  
25 (e.g., residues 43-139 of Seq. ID No. 5). These most preferred sequences include both allelic and species variants of the OP-1 and OP-2 proteins, including the Drosophila 60A protein. Accordingly, in another preferred aspect of the invention, useful morphogens  
30 include active proteins comprising species of polypeptide chains having the generic amino acid sequence herein referred to as "OPX", which accommodates the homologies between the various identified species of OP1 and OP2 (Seq. ID No. 29).

The morphogens useful in the methods, composition and devices of this invention include proteins comprising any of the polypeptide chains described above, whether isolated from naturally-occurring sources, or produced by recombinant DNA or other synthetic techniques, and includes allelic and species variants of these proteins, naturally-occurring or biosynthetic mutants thereof, as well as various truncated and fusion constructs. Deletion or addition mutants also are envisioned to be active, including those which may alter the conserved C-terminal cysteine skeleton, provided that the alteration does not functionally disrupt the relationship of these cysteines in the folded structure. Accordingly, such active forms are considered the equivalent of the specifically described constructs disclosed herein. The proteins may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated or mutated forms of native or biosynthetic proteins, produced by expression of recombinant DNA in host cells.

The morphogenic proteins can be expressed from intact or truncated cDNA or from synthetic DNAs in procaryotic or eucaryotic host cells, and purified, cleaved, refolded, and dimerized to form morphogenically active compositions. Currently preferred host cells include E. coli or mammalian cells, such as CHO, COS or BSC cells. A detailed description of the morphogens useful in the methods, compositions and devices of this invention is disclosed in copending US patent application Serial Nos. 752,764, filed August 30, 1991, and 667,274, filed March 11, 1991, the disclosure of which are incorporated herein by reference.

Thus, in view of this disclosure, skilled genetic engineers can isolate genes from cDNA or genomic libraries of various different species which encode  
5 appropriate amino acid sequences, or construct DNAs from oligonucleotides, and then can express them in various types of host cells, including both procaryotes and eucaryotes, to produce large quantities of active proteins capable of maintaining the integrity of the  
10 gastrointestinal tract luminal lining in individuals at risk for ulcer formation.

The foregoing and other objects, features and advantages of the present invention will be made more  
15 apparent from the following detailed description of the invention.

### Brief Description of the Drawings

The foregoing and other objects and features of  
5 this invention, as well as the invention itself, may be  
more fully understood from the following description,  
when read together with the accompanying drawings, in  
which:

10 Fig. 1 graphs the effect of a morphogen (e.g., OP-  
1) and a placebo control on mucositic lesion formation;

Fig. 2(A and B) are photomicrographs illustrating  
the ability of morphogens to inhibit lesion formation  
15 in an oral mucositis animal model, where (2A) shows  
lesion formation in untreated hamster cheek pouches;  
and (2B) shows the significantly reduced effect on  
morphogen treated cheek pouches;

20 Fig. 3(A and B) graphs the antiproliferative effect of  
morphogens on mink lung cells; and

Fig. 4(A-D) graphs the effects of a morphogen (eg.,  
OP-1, Figs. 4A and 4C) and TGF- $\beta$  (Fig. 4B and 4D) on  
25 collagen (4A and 4B) and hyaluronic acid (4C and 4D)  
production in primary fibroblast cultures.

### Detailed Description of the Invention

It now has been discovered that the proteins described herein are effective agents for maintaining the integrity of the gastrointestinal tract luminal lining in a mammal. As described herein, these proteins ("morphogens") are capable of substantially inhibiting lesion formation in the basal epithelium, as well as stimulating the repair and regeneration the barrier tissue following ulceration. The proteins are capable of inhibiting epithelial cell proliferation and protecting the barrier tissue from damage. The proteins also are capable of inhibiting scar tissue formation that typically follows lesion formation in a mammal. In addition, the morphogens also can inhibit the inflammation normally associated with ulcerative diseases. The proteins may be used to treat ulcerative diseases of the gastrointestinal tract, including oral mucositis, peptic ulcers, ulcerative colitis, proctitis, and regional enteritis. The proteins also may be used to protect and/or treat GI tissue subject to damage in a xerostomaic individual. Finally, the morphogens may be administered as part of a chemical or radiotherapy to inhibit lesion formation in a patient undergoing cancer therapy and enhance the efficacy of the therapy thereby.

Provided below are detailed descriptions of suitable morphogens useful in the methods and compositions of this invention, as well as methods for their administration and application, and numerous, nonlimiting examples which demonstrate (1) the suitability of the morphogens described herein as therapeutic agents for maintaining the integrity of the gastrointestinal tract luminal lining, and (2) provide

assays with which to test candidate morphogens and morphogen-stimulating agents for their efficacy. Specifically, the examples demonstrate the ability of morphogens to treat oral mucositis, duodenal ulcers, peptic ulcers, and ulcerative colitis (Examples 2-5), inhibit epithelial cell proliferation (Example 6), inhibit inflammation (Example 7) and inhibit scar tissue formation (Example 8.) The Examples also describe methods for identifying morphogen-expressing tissue and screening for candidate morphogen stimulating agents (Examples 1, 2 and 10.)

#### I. Useful Morphogens

As defined herein a protein is morphogenic if it is capable of inducing the developmental cascade of cellular and molecular events that culminate in the formation of new, organ-specific tissue and comprises at least the conserved C-terminal six cysteine skeleton or its functional equivalent (see supra). Details of how the morphogens useful in the method of this invention first were identified, as well as a description on how to make, use and test them for morphogenic activity, are disclosed in USSN 667,274, filed March 11, 1991 and USSN 752,764, filed August 30, 1991, the disclosures of which are hereby incorporated by reference. As disclosed therein, the morphogens may be purified from naturally-sourced material or recombinantly produced from procaryotic or eucaryotic host cells, using the genetic sequences disclosed therein. Alternatively, novel morphogenic sequences may be identified following the procedures disclosed therein.

Particularly useful proteins include those which  
comprise the naturally derived sequences disclosed in  
Table II. Other useful sequences include 60A, BMP5,  
BMP6, BMP3, and biosynthetic constructs such as those  
5 disclosed in U.S. Pat. 5,011,691, the disclosure of  
which is incorporated herein by reference (e.g., COP-1,  
COP-3, COP-4, COP-5, COP-7, and COP-16).

Accordingly, the morphogens useful in the methods  
10 and compositions of this invention also may be  
described by morphogenically active proteins having  
amino acid sequences sharing 70% or, preferably, 80%  
homology (similarity) with any of the sequences  
described above, where "homology" is as defined herein  
15 above.

The morphogens useful in the method of this  
invention also can be described by any of the 6 generic  
sequences described herein (Generic Sequences 1, 2, 3,  
20 4, 5 and 6). Generic sequences 1 and 2 also may  
include, at their N-terminus, the sequence

Cys Xaa Xaa Xaa Xaa (Seq. ID No. 15)

1

5

25

Table II, set forth below, compares the amino acid  
sequences of the active regions of native proteins that  
have been identified as morphogens, including human  
OP-1 (hOP-1, Seq. ID Nos. 5 and 16-17), mouse OP-1  
30 (mOP-1, Seq. ID Nos. 6 and 18-19), human and mouse OP-2  
(Seq. ID Nos. 7, 8, and 20-23), CBMP2A (Seq. ID No. 9),  
CBMP2B (Seq. ID No. 10), DPP (from Drosophila, Seq. ID  
No. 11), Vgl, (from Xenopus, Seq. ID No. 12), Vgr-1  
(from mouse, Seq. ID No. 13), and GDF-1 (from mouse,  
35 Seq. ID No. 14.) The sequences are aligned essentially

following the method of Needleman et al. (1970) J. Mol. Biol., 48:443-453, calculated using the Align Program (DNASTar, Inc.) In the table, three dots indicates that the amino acid in that position is the same as the amino acid in hOP-1. Three dashes indicates that no amino acid is present in that position, and are included for purposes of illustrating homologies. For example, amino acid residue 60 of CBMP-2A and CBMP-2B is "missing". Of course, both these amino acid sequences in this region comprise Asn-Ser (residues 58, 59), with CBMP-2A then comprising Lys and Ile, whereas CBMP-2B comprises Ser and Ile.

TABLE II

hOP-1	Cys	Lys	Lys	His	Glu	Leu	Tyr	Val	
mOP-1	...	...	...	...	...	...	...	...	
hOP-2	...	Arg	Arg	...	...	...	...	...	
mOP-2	...	Arg	Arg	...	...	...	...	...	
DPP	...	Arg	Arg	...	Ser	...	...	...	
Vgl	...	...	Lys	Arg	His	...	...	...	
Vgr-1	...	...	...	...	Gly	...	...	...	
CBMP-2A	...	...	Arg	...	Pro	...	...	...	
CBMP-2B	...	Arg	Arg	...	Ser	...	...	...	
GDF-1	...	Arg	Ala	Arg	Arg	...	...	...	
	1				5				
hOP-1	Ser	Phe	Arg	Asp	Leu	Gly	Trp	Gln	Asp
mOP-1	...	...	...	...	...	...	...	...	...
hOP-2	...	...	Gln	...	...	...	...	Leu	...
mOP-2	Ser	...	...	...	...	...	...	Leu	...
DPP	Asp	...	Ser	...	Val	...	...	Asp	...
Vgl	Glu	...	Lys	...	Val	...	...	...	Asn



5	Vgr-1	...	...	Gln	...	Val	...	...	...	...
	CBMP-2A	Asp	...	Ser	...	Val	...	...	Asn	...
	CBMP-2B	Asp	...	Ser	...	Val	...	...	Asn	...
	GDF-1	...	...	...	Glu	Val	...	...	His	Arg
			10					15		
10	hOP-1	Trp	Ile	Ile	Ala	Pro	Glu	Gly	Tyr	Ala
	mOP-1	...	...	...	...	...	...	...	...	...
	hOP-2	...	Val	...	...	...	Gln	...	...	Ser
	mOP-2	...	Val	...	...	...	Gln	...	...	Ser
	DPP	...	...	Val	...	...	Leu	...	...	Asp
	Vgl	...	Val	...	...	...	Gln	...	...	Met
15	Vgr-1	...	...	...	...	...	Lys	...	...	...
	CBMP-2A	...	...	Val	...	...	Pro	...	...	His
	CBMP-2B	...	...	Val	...	...	Pro	...	...	Gln
	GDF-1	...	Val	...	...	...	Arg	...	Phe	Leu
			20					25		
20	hOP-1	Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ala
	mOP-1	...	...	...	...	...	...	...	...	...
	hOP-2	...	...	...	...	...	...	...	...	Ser
	mOP-2	...	...	...	...	...	...	...	...	...
	DPP	...	...	...	...	His	...	Lys	...	Pro
	Vgl	...	Asn	...	...	Tyr	...	...	...	Pro
25	Vgr-1	...	Asn	...	...	Asp	...	...	...	Ser
	CBMP-2A	...	Phe	...	...	His	...	Glu	...	Pro
	CBMP-2B	...	Phe	...	...	His	...	Asp	...	Pro
	GDF-1	...	Asn	...	...	Gln	...	Gln	...	...
			30					35		
35	hOP-1	Phe	Pro	Leu	Asn	Ser	Tyr	Met	Asn	Ala
	mOP-1	...	...	...	...	...	...	...	...	...
	hOP-2	...	...	...	Asp	...	Cys	...	...	...
	mOP-2	...	...	...	Asp	...	Cys	...	...	...

[illegible]

	hOP-2	...	...	Ala	...	...	...	...	...	Lys
	mOP-2	...	...	Ala	...	...	...	...	...	Lys
	DPP	...	...	Ala	...	...	Val	...	...	...
	Vgl	...	Leu	...	...	...	Val	...	...	Lys
5	Vgr-1	...	...	...	...	...	...	...	...	Lys
	CBMP-2A	...	...	Ala	...	...	Val	...	...	Glu
	CBMP-2B	...	...	Ala	...	...	Val	...	...	Glu
	GDF-1	Asp	Leu	...	...	...	Val	...	Ala	Arg
				65					70	

10

	hOP-1	Leu	Asn	Ala	Ile	Ser	Val	Leu	Tyr	Phe
	mOP-1	...	...	...	...	...	...	...	...	...
	hOP-2	...	Ser	...	Thr	...	...	...	...	Tyr
15	mOP-2	...	Ser	...	Thr	...	...	...	...	Tyr
	Vgl	Met	Ser	Pro	...	...	Met	...	Phe	Tyr
	Vgr-1	Val	...	...	...	...	...	...	...	...
	DPP	...	Asp	Ser	Val	Ala	Met	...	...	Leu
	CBMP-2A	...	Ser	...	...	...	Met	...	...	Leu
20	CBMP-2B	...	Ser	...	...	...	Met	...	...	Leu
	GDF-1	...	Ser	Pro	...	...	...	...	Phe	...
					75					80

	hOP-1	Asp	Asp	Ser	Ser	Asn	Val	Ile	Leu	Lys
	mOP-1	...	...	...	...	...	...	...	...	...
25	hOP-2	...	Ser	...	Asn	...	...	...	...	Arg
	mOP-2	...	Ser	...	Asn	...	...	...	...	Arg
	DPP	Asn	...	Gln	...	Thr	...	Val	...	...
	Vgl	...	Asn	Asn	Asp	...	...	Val	...	Arg
	Vgr-1	...	...	Asn	...	...	...	...	...	...
30	CBMP-2A	...	Glu	Asn	Glu	Lys	...	Val	...	...
	CBMP-2B	...	Glu	Tyr	Asp	Lys	...	Val	...	...
	GDF-1	...	Asn	...	Asp	...	...	Val	...	Arg

85

35

	hOP-1	Lys	Tyr	Arg	Asn	Met	Val	Val	Arg
	mOP-1	...	...	...	...	...	...	...	...
	hOP-2	...	His	...	...	...	...	...	Lys
	mOP-2	...	His	...	...	...	...	...	Lys
5	DPP	Asn	...	Gln	Glu	...	Thr	...	Val
	Vgl	His	...	Glu	...	...	Ala	...	Asp
	Vgr-1	...	...	...	...	...	...	...	...
	CBMP-2A	Asn	...	Gln	Asp	...	...	...	Glu
	CBMP-2B	Asn	...	Gln	Glu	...	...	...	Glu
10	GDF-1	Gln	...	Glu	Asp	...	...	...	Asp
		90					95		

	hOP-1	Ala	Cys	Gly	Cys	His
15	mOP-1	...	...	...	...	...
	hOP-2	...	...	...	...	...
	mOP-2	...	...	...	...	...
	DPP	Gly	...	...	...	Arg
	Vgl	Glu	...	...	...	Arg
20	Vgr-1	...	...	...	...	...
	CBMP-2A	Gly	...	...	...	Arg
	CBMP-2B	Gly	...	...	...	Arg
	GDF-1	Glu	...	...	...	Arg

100

25    \*\*Between residues 43 and 44 of GDF-1 lies the amino acid  
sequence Gly-Gly-Pro-Pro.

As is apparent from the foregoing amino acid  
sequence comparisons, significant amino acid changes  
30 can be made within the generic sequences while  
retaining the morphogenic activity. For example, while  
the GDF-1 protein sequence depicted in Table II shares  
only about 50% amino acid identity with the hOP1  
sequence described therein, the GDF-1 sequence shares  
35 greater than 70% amino acid sequence homology (or

"similarity") with the hOP1 sequence, where "homology" or "similarity" includes allowed conservative amino acid changes within the sequence as defined by Dayoff, et al., Atlas of Protein Sequence and Structure vol.5, 5 supp.3, pp.345-362, (M.O. Dayoff, ed., Nat'l BioMed. Res. Fd'n, Washington D.C. 1979.)

10 The currently most preferred protein sequences useful as morphogens in this invention include those having greater than 60% identity, preferably greater than 65% identity, with the amino acid sequence defining the conserved six cysteine skeleton of hOP1 (e.g., residues 43-139 of Seq. ID No. 5). These most preferred sequences include both allelic and species 15 variants of the OP-1 and OP-2 proteins, including the Drosophila 60A protein. Accordingly, in still another preferred aspect, the invention includes morphogens comprising species of polypeptide chains having the generic amino acid sequence referred to herein as 20 "OPX", which defines the seven cysteine skeleton and accommodates the identities between the various identified mouse and human OP1 and OP2 proteins. OPX is presented in Seq. ID No. 29. As described therein, each Xaa at a given position independently is selected 25 from the residues occurring at the corresponding position in the C-terminal sequence of mouse or human OP1 or OP2 (see Seq. ID Nos. 5-8 and/or Seq. ID Nos. 16-23).

## II. Formulations and Methods for Administering Therapeutic Agents

The morphogens or morphogen-stimulating agents may  
5 be provided to an individual by any suitable means,  
preferably by oral, rectal or other direct  
administration or, alternatively, by systemic  
administration.

10 The suitability of systemic administration is  
demonstrated by the detection of endogenous morphogen  
in milk and human serum described, for example, in  
copending USSN 923,780, filed July 31, 1992,  
incorporated herein by reference, and in Example 2,  
15 below. Where the morphogen is to be provided  
parenterally, such as by intravenous, subcutaneous,  
intramuscular, intraorbital, intraventricular,  
intracapsular, intraspinal, intracisternal,  
intraperitoneal or vaginal administration, the  
20 morphogen preferably comprises part of an aqueous  
solution. The solution is physiologically acceptable  
so that in addition to delivery of the desired  
morphogen to the patient, the solution does not  
otherwise adversely affect the patient's electrolyte  
25 and volume balance. The aqueous medium for the  
morphogen thus may comprise normal physiologic saline  
(0.85% NaCl, 0.15M), pH 7-7.4. The aqueous solution  
containing the morphogen can be made, for example, by  
dissolving the protein in 50% ethanol containing  
30 acetonitrile in 0.1% trifluoroacetic acid (TFA) or 0.1%  
HCl, or equivalent solvents. One volume of the  
resultant solution then is added, for example, to ten  
volumes of phosphate buffered saline (PBS), which  
further may include 0.1-0.2% human serum albumin (HSA).  
35 The resultant solution preferably is vortexed

extensively. If desired, a given morphogen may be made more soluble by association with a suitable molecule.

For example, the pro form of the morphogenic protein comprises a species that is soluble in physiological solutions. In fact, the endogenous protein is thought to be transported (e.g., secreted and circulated) in this form. This soluble form of the protein may be obtained from the culture medium of morphogen-secreting cells. Alternatively, a soluble species may be

formulated by complexing the mature dimer, (or an active fragment thereof) with part or all of a pro domain. Other components, including various serum proteins, also may be useful.

Useful solutions for parenteral administration may be prepared by any of the methods well known in the pharmaceutical art, described, for example, in Remington's Pharmaceutical Sciences (Gennaro, A., ed.), Mack Pub., 1990. Formulations may include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Other potentially useful parenteral delivery systems for these morphogens include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for parenteral administration may also include cutric acid for vaginal administration.

Preferably, the morphogens described herein are administered directly e.g., topically, for example, by oral or rectal administration, or by directly applying the therapeutic formulation onto the desired tissue. Oral administration of proteins as therapeutics generally is not practiced as most proteins are readily

degraded by digestive enzymes and acids in the mammalian digestive system before they can be absorbed into the bloodstream. However, the morphogens described herein typically are acid stable and protease-resistant (see, for example, U.S. Pat.No. 4,968,590.) In addition, at least one morphogen, OP-1, has been identified in mammary gland extract, colostrum and 57-day milk. Moreover, the OP-1 purified from mammary gland extract is morphogenically active.

Specifically, this protein induces endochondral bone formation in mammals when implanted subcutaneously in association with a suitable matrix material, using a standard in vivo bone assay, such as is disclosed in U.S. Pat.No. 4,968,590. Moreover, as described above, the morphogen also is detected in the bloodstream. These findings indicate that oral administration is a viable means for administering morphogens to an individual. In addition, while the mature forms of certain morphogens described herein typically are sparingly soluble, the morphogen form found in milk (and mammary gland extract and colostrum) is readily soluble, probably by association of the mature, morphogenically active form with part or all of the pro domain of the intact sequence and/or by association with one or more milk components. Accordingly, the compounds provided herein also may be associated with molecules capable of enhancing their solubility in vitro or in vivo.

For oral mucositis treatments the morphogens or morphogen-stimulating agents (herein below referred to collectively as "therapeutic agent") may be formulated into an oral rinse similar to a mouthwash, where the liquid is swished around in the mouth so that the therapeutic agent is brought in contact with the oral



mucosa to maximize treatment of lesions.

Alternatively, the therapeutic agent may be formulated as part of a slow dissolving troche or lozenge, or dispersed in a gum base suitable for a chewing gum,  
5 such that the agent is released with mastication.

Longer contact with the mucosal surface of the mouth cavity or elsewhere in the G.I. tract can be attained by direct topical administration, using a  
10 suitable vehicle which is capable of coating mucosa. Typical examples are pectin-containing formulations or sucralfate suspensions, such as are found in Kaopectate and Milk of Magnesia. Formulations for direct administration also may include glycerol and other  
15 compositions of high viscosity. Tissue adhesives capable of adhering to the mucosal tissue surface and maintaining the therapeutic agent at the tissue locus also may be used. Useful adhesive compositions include hydroxypropyl-cellulose-containing solutions, such as  
20 is found in Orabase<sup>R</sup> (Colgate-Hoyt Laboratories, Norwood, MA), or fibrinogen/thrombin-containing solutions. Another useful adhesive is the bio-adhesive described in copending USSN 627,323, incorporated herein above by reference. Preferably these  
25 formulations are painted onto the tissue surface or formulated as an aerosol and sprayed onto the tissue surface. As for parenteral administration, the therapeutic agent may be associated with a molecule that enhances solubility. For example, addition of  
30 0.2% casein increases solubility of the mature active form of OP-1 by 80%. Another useful molecule is a morphogen pro domain.

For all treatments of the gastrointestinal tract, the therapeutic agent also may be formulated into a solid or liquid to be consumed or as an inhalant. For treatments of the lower bowel, formulations for rectal administration may be preferable, and may include suppositories, creams, gels, lotions and the like.

In all applications, biocompatible, preferably bioresorbable, polymers, including, for example, hyaluronic acid, collagen, polybutyrate, tricalcium phosphate, glycolide, lactide and lactide/glycolide copolymers, also may be useful excipients to control the release of the morphogen in vivo. Tablets or capsules may be prepared by employing additives such as pharmaceutically acceptable carriers (e.g., lactose, corn starch, light silicic anhydride, microcrystalline cellulose, sucrose), binders (e.g., alpha-form starch, methylcellulose, carboxymethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose, polyvinylpyrrolidone), disintegrating agents (e.g., carboxymethylcellulose calcium, starch, low substituted hydroxypropylcellulose), surfactants [e.g., Tween 80 Kao-Atlas), Pluronic F68 (Asahi Denka, Japan); polyoxyethylene-polyoxypropylene copolymer)], antioxidants (e.g., L-cysteine, sodium sulfite, sodium ascorbate), lubricants (e.g., magnesium stearate, talc), and the like.

Formulations for inhalation administration may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for rectal administration

also may include methoxy salicylate. The formulations for rectal administration also can be a spreadable cream, gel, suppository, foam, lotion or ointment having a pharmaceutically acceptable nontoxic vehicle or carrier. Biocompatible, preferably bioresorbable, polymers, including, for example, hyaluronic acid, collagen, polybutyrate, tricalcium phosphate, lactide and lactide/glycolide copolymers, also may be useful excipients to control the release of the morphogen in vivo.

The compounds provided herein also may be associated with molecules capable of targeting the morphogen or morphogen-stimulating agent to the gastrointestinal barrier tissue. For example, an antibody, antibody fragment, or other binding protein that interacts specifically with a surface molecule on basal epithelial cells, may be used. Useful targeting molecules may be designed, for example, using the single chain binding site technology disclosed, for example, in U.S. Pat. No. 5,091,513.

As described above, the morphogens provided herein share significant sequence homology in the C-terminal active domains. By contrast, the sequences typically diverge significantly in the sequences which define the pro domain. Accordingly, the pro domain is thought to be morphogen-specific. As described above, it is also known that the various morphogens identified to date are differentially expressed in different tissues. Accordingly, without being limited to any given theory, it is likely that, under natural conditions in the body, selected morphogens typically act on a given tissue. Accordingly, part or all of the pro domains which have been identified associated with the active

form of the morphogen in solution, may serve as targeting molecules for the morphogens described herein. For example, the pro domains may interact specifically with one or more molecules at the target  
5 tissue to direct the morphogen associated with the pro domain to that tissue. Accordingly, another useful targeting molecule for targeting a morphogen to gastrointestinal barrier tissues may include part or all of a morphogen pro domain, particularly part or all  
10 of a pro domain normally associated with an endogenous morphogen known to act on GI tract tissue. As described above, morphogen species comprising the pro domain may be obtained from the culture medium of morphogen-secreting mammalian cells. Alternatively, a  
15 tissue-targeting species may be formulated by complexing the mature dimer (or an active fragment thereof) with part or all of a pro domain. Example 1 describes a protocol for identifying morphogen-expressing tissue and/or morphogen target  
20 tissue.

Finally, the morphogens or morphogen-stimulating agents provided herein may be administered alone or in combination with other molecules known to be beneficial  
25 in treating gastrointestinal tract ulcers, particularly symptom-alleviating cofactors. Useful pharmaceutical cofactors include analgesics and anesthetics such as xylocaine, benzocaine and the like; antiseptics such as chlorohexidine; anti-viral and anti-fungal agents; and  
30 antibiotics, including aminoglycosides, macrolides, penicillins, and cephalosporins. Other potentially useful cofactors include antisecretory agents such as H2-receptor antagonists (e.g., cimetidine, ranitidine, famotidine, roxatidine acetate), muscarine receptor  
35 antagonists (e.g., Pirenzepine), and antacids such as

aluminum hydroxide gel, magnesium hydroxide and sodium bicarbonate. Such agents may be administered either separately or as components of the therapeutic composition containing morphogens or morphogen-stimulating agents.

The compositions can be formulated for parenteral or direct administration to humans or other mammals in therapeutically effective amounts, e.g., amounts which provide appropriate concentrations for a time sufficient to protect the patient's gastrointestinal luminal lining from lesion formation, including amounts which limit the proliferation of epithelial cells, particularly the basal epithelial cells of the G.I. tract, amounts which limit the inflammation associated with the ulcerative diseases and disorders described above, and amounts sufficient to stimulate lesion repair and tissue regeneration.

As will be appreciated by those skilled in the art, the concentration of the compounds described in a therapeutic composition will vary depending upon a number of factors, including the dosage of the drug to be administered, the chemical characteristics (e.g., hydrophobicity) of the compounds employed, and the route of administration. The preferred dosage of drug to be administered also is likely to depend on such variables as the type and extent of progression of the ulcerative disease, the overall health status of the particular patient, the relative biological efficacy of the compound selected, the formulation of the compound excipients, and its route of administration. In general terms, the compounds of this invention may be provided in an aqueous physiological buffer solution containing about 0.001 to 10% w/v compound for parenteral

administration. Typical dose ranges are from about 10  
ng/kg to about 1 g/kg of body weight per day; a  
preferred dose range is from about 0.1  $\mu$ g/kg to  
100 mg/kg of body weight per day. Optimally, the  
5 morphogen dosage given is between 0.1-100  $\mu$ g of protein  
per kilogram weight of the patient. Administration may  
be a single dose per day, or may include multiple  
doses, such as multiple rinsings with a mouthwash,  
e.g., a 1 minute rinse three or four times daily. No  
10 obvious induced pathological lesions are induced when  
mature morphogen (e.g., OP-1, 20  $\mu$ g) is administered  
daily to normal growing rats for 21 consecutive days.  
Moreover, 10  $\mu$ g systemic injections of morphogen (e.g.,  
OP-1) injected daily for 10 days into normal newborn  
15 mice does not produce any gross abnormalities.

In administering morphogens systemically in the  
methods of the present invention, preferably a large  
volume loading dose is used at the start of the  
20 treatment. The treatment then is continued with a  
maintenance dose. Further administration then can be  
determined by monitoring at intervals the levels of the  
morphogen in the blood using, for example, a morphogen-  
specific antibody and standard immunoassay procedures.

25 Where injury to the mucosa is induced deliberately  
or incidentally, as part of, for example, a chemical or  
radiation therapy, the morphogen preferably is provided  
just prior to, or concomitant with induction of the  
30 treatment. Preferably, the morphogen is administered  
prophylactically in a clinical setting. Optimally, the  
morphogen dosage given is between 0.1-100  $\mu$ g of protein

per kilogram weight of the patient. Similarly, the morphogen may be administered prophylactically to individuals at risk for ulcer formation, including xerostomatic or immune-compromised individuals,  
5 regardless of etiology.

An effective amount of an agent capable of stimulating endogenous morphogen levels also may be administered by any of the routes described above. For  
10 example, an agent capable of stimulating morphogen production in and/or secretion to G.I. tract tissue cells may be provided to a mammal. A method for identifying and testing agents capable of modulating the levels of endogenous morphogens in a given tissue  
15 is described generally herein in Example 10, and in detail in copending USSN [CRP059CP], filed August 28, 1992, and USSN 752,859, filed August 30, 1991, the disclosures of which are incorporated herein by reference. In addition, Example 1 describes a protocol  
20 for determining morphogen-expressing tissue. Briefly, candidate compounds can be identified and tested by incubating the compound in vitro with a test tissue or cells thereof, for a time sufficient to allow the compound to affect the production, i.e., the expression  
25 and/or secretion, of a morphogen produced by the cells of that tissue. Here, suitable tissue or cultured cells of a tissue preferably would include cells of the G.I. tract barrier. For example, suitable tissue for testing may include cultured cells isolated from the  
30 basal epithelium and mucosa, and the like.

A currently preferred detection means for evaluating the level of the morphogen in culture upon exposure to the candidate compound comprises an  
35 immunoassay utilizing an antibody or other suitable

binding protein capable of reacting specifically with a morphogen and being detected as part of a complex with the morphogen. Immunoassays may be performed using standard techniques known in the art and antibodies raised against a morphogen and specific for that morphogen. As described herein, morphogens may be isolated from natural-sourced material or they may be recombinantly produced. Agents capable of stimulating endogenous morphogens then may formulated into pharmaceutical preparations and administered as described herein.

### III. Examples

#### 15 Example 1. Identification of Morphogen-Expressing Tissue

Determining the tissue distribution of morphogens may be used to identify different morphogens expressed in a given tissue, as well as to identify new, related morphogens. Tissue distribution also may be used to identify useful morphogen-producing tissue for use in screening and identifying candidate morphogen-stimulating agents. The morphogens (or their mRNA transcripts) readily are identified in different tissues using standard methodologies and minor modifications thereof in tissues where expression may be low. For example, protein distribution may be determined using standard Western blot analysis or immunofluorescent techniques, and antibodies specific to the morphogen or morphogens of interest. Similarly, the distribution of morphogen transcripts may be determined using standard Northern hybridization protocols and transcript-specific oligonucleotide probes.



Any probe capable of hybridizing specifically to a transcript, and distinguishing the transcript of interest from other, related transcripts may be used. Because the morphogens described herein share such high sequence homology in their active, C-terminal domains, the tissue distribution of a specific morphogen transcript may best be determined using a probe specific for the pro region of the immature protein and/or the N-terminal region of the mature protein.

Another useful sequence is the 3' non-coding region flanking and immediately following the stop codon. These portions of the sequence vary substantially among the morphogens of this invention, and accordingly, are specific for each protein. For example, a particularly useful Vgr-1-specific probe sequence is the PvuII-SacI fragment, a 265 bp fragment encoding both a portion of the untranslated pro region and the N-terminus of the mature sequence (see Lyons et al. (1989) PNAS 86:4554-4558 for a description of the cDNA sequence).

Similarly, particularly useful mOP-1-specific probe sequences are the BstXI-BglI fragment, a 0.68 Kb sequence that covers approximately two-thirds of the mOP-1 pro region; a StuI-StuI fragment, a 0.2 Kb sequence immediately upstream of the 7-cysteine domain; and the Earl-PstI fragment, an 0.3 Kb fragment containing a portion of the 3'untranslated sequence (See Seq. ID No. 18, where the pro region is defined essentially by residues 30-291.) Similar approaches may be used, for example, with hOP-1 (Seq. ID No. 16) or human or mouse OP-2 (Seq. ID Nos. 20 and 22.)

Using these morphogen-specific probes, which may be synthetically engineered or obtained from cloned sequences, morphogen transcripts can be identified in mammalian tissue, using standard methodologies well

known to those having ordinary skill in the art. Briefly, total RNA is prepared from various adult murine tissues (e.g., liver, kidney, testis, heart, brain, thymus and stomach) by a standard methodology such as by the method of Chomczyaski et al. ((1987) Anal. Biochem 162:156-159) and described below. Poly (A)+ RNA is prepared by using oligo (dT)-cellulose chromatography (e.g., Type 7, from Pharmacia LKB Biotechnology, Inc.). Poly (A)+ RNA (generally 15  $\mu$ g) from each tissue is fractionated on a 1% agarose/formaldehyde gel and transferred onto a Nytran membrane (Schleicher & Schuell). Following the transfer, the membrane is baked at 80°C and the RNA is cross-linked under UV light (generally 30 seconds at 1 mW/cm<sup>2</sup>). Prior to hybridization, the appropriate probe is denatured by heating. The hybridization is carried out in a lucite cylinder rotating in a roller bottle apparatus at approximately 1 rev/min for approximately 15 hours at 37°C using a hybridization mix of 40% formamide, 5 x Denhardtts, 5 x SSPE, and 0.1% SDS. Following hybridization, the non-specific counts are washed off the filters in 0.1 x SSPE, 0.1% SDS at 50°C.

Examples demonstrating the tissue distribution of various morphogens, including Vgr-1, OP-1, BMP2, BMP3, BMP4, BMP5, GDF-1, and OP-2 in developing and adult tissue are disclosed in co-pending USSN 752,764, and in Ozkaynak, et al., (1991) Biochem. Biophys. Res. Commn. 179:116-123, and Ozkaynak, et al. (1992) (JBC, in press), the disclosures of which are incorporated herein by reference. Using the general probing methodology described herein, Northern blot hybridizations using probes specific for these morphogens to probe brain, spleen, lung, heart, liver and kidney tissue indicate that kidney-related tissue

appears to be the primary expression source for OP-1, with brain, heart and lung tissues being secondary sources. OP-1 mRNA also was identified in salivary glands, specifically rat parotid glands, using this  
5 probing methodology. Lung tissue appears to be the primary tissue expression source for Vgr-1, BMP5, BMP4 and BMP3. Lower levels of Vgr-1 also are seen in kidney and heart tissue, while the liver appears to be a secondary expression source for BMP5, and the spleen  
10 appears to be a secondary expression source for BMP4. GDF-1 appears to be expressed primarily in brain tissue. To date, OP-2 appears to be expressed primarily in early embryonic tissue. Specifically, Northern blots of murine embryos and 6-day post-natal  
15 animals shows abundant OP2 expression in 8-day embryos. Expression is reduced significantly in 17-day embryos and is not detected in post-natal animals.

Immunolocalization studies using OP-1 specific  
20 antibodies also localize the morphogen to both the inner circular and outer longitudinal coats of smooth muscles in the tubular organs of the digestive system during early embryo development (gestation: weeks 5-13), suggesting the endogenous morphogen also plays a  
25 role in tissue morphogenesis of the digestive tract.

Moreover, Northern blot analysis on rat tissue (probed with an mOP-1-specific labelled nucleotide fragment, as described above) identifies OP-1 mRNA in  
30 the gastrointestinal tract tissues of growing rats, including the stomach, duodenal and intestine tissues. These data demonstrate that morphogens are both expressed in, and act on, tissues of the GI tract.

## Example 2. Active Morphogens in Body Fluids

OP-1 expression has been identified in saliva (specifically, the rat parotid gland, see Example 1),  
5 human blood serum, and various milk forms, including mammary gland extract, colostrum, and 57-day bovine milk. Moreover, and as described in USSN 923,780, the disclosure of which is incorporated hereinabove by reference, the body fluid-extracted protein is  
10 morphogenically active. The discovery that the morphogen naturally is present in milk and saliva, together with the known observation that mature, active OP-1 is acid-stable and protease-resistant, indicate that oral administration is a useful route for  
15 therapeutic administration of morphogen to a mammal. Oral administration typically is the preferred mode of delivery for extended or prophylactic therapies. In addition, the identification of morphogen in all milk forms, including colostrum, suggests that the protein  
20 may play a significant role in tissue development, including skeletal development, of juveniles.

### 2.1 Morphogen Detection in Milk

25 OP-1 was partially purified from rat mammary gland extract and bovine colostrum and 57 day milk by passing these fluids over a series of chromatography columns: (e.g., cation-exchange, affinity and reverse phase). At each step the eluant was collected in fractions and  
30 these were tested for the presence of OP-1 by standard immunoblot. Immunoreactive fractions then were combined and purified further. The final, partially purified product then was examined for the presence of OP-1 by Western blot analysis using OP-1-specific  
35 antisera, and tested for in vivo and in vitro activity.

OP-1 purified from the different milk sources were characterized by Western blotting using antibodies raised against OP-1 and BMP2. Antibodies were prepared using standard immunology protocols well known in the art, and as described generally in Example 15, below, using full-length E. coli-produced OP-1 and BMP2 as the immunogens. In all cases, the purified OP-1 reacted only with the anti-OP-1 antibody, and not with anti-BMP2 antibody.

10

The morphogenic activity of OP-1 purified from mammary gland extract was evaluated in vivo essentially following the rat model assay described in U.S. Pat. No. 4,968,590, hereby incorporated by reference. Briefly, a sample was prepared from each OP-1 immunoreactive fraction of the mammary gland extract-derived OP-1 final product by lyophilizing a portion (33%) of the fraction and resuspending the protein in 220 $\mu$ l of 50% acetonitrile/0.1% TFA. After vortexing, 25 mg of collagen matrix was added. The samples were lyophilized overnight, and implanted in Long Evans rats (Charles River Laboratories, Wilmington, MA, 28-35 days old). Each fraction was implanted in duplicate. For details of the collagen matrix implantation procedure, see, for example, U.S. Pat. No. 4,968,590, hereby incorporated by reference. After 12 days, the implants were removed and evaluated for new bone formation by histological observation as described in U.S. Patent No. 4,968,590. In all cases, the immunoreactive fractions were osteogenically active.

## 2.2 Morphogen Detection in Serum

Morphogen may be detected in serum using morphogen-specific antibodies. The assay may be performed using  
5 any standard immunoassay, such as Western blot (immunoblot) and the like. Preferably, the assay is performed using an affinity column to which the morphogen-specific antibody is bound and through which the sample serum then is poured, to selectively extract  
10 the morphogen of interest. The morphogen then is eluted. A suitable elution buffer may be determined empirically by determining appropriate binding and elution conditions first with a control (e.g., purified, recombinantly-produced morphogen.) Fractions  
15 then are tested for the presence of the morphogen by standard immunoblot, and the results confirmed by N-terminal sequencing. Preferably, the affinity column is prepared using monoclonal antibodies. Morphogen concentrations in serum or other fluid samples then may  
20 be determined using standard protein quantification techniques, including by spectrophotometric absorbance or by quantitation of conjugated antibody.

Presented below is a sample protocol for  
25 identifying OP-1 in serum. Following this general methodology other morphogens may be detected in body fluids, including serum. The identification of morphogen in serum further indicates that systemic administration is a suitable means for providing  
30 therapeutic concentrations of a morphogen to an individual, and that morphogens likely behave systemically as endocrine-like factors. Finally, using this protocol, fluctuations in endogenous morphogen levels can be detected, and these altered levels may be  
35 used as an indicator of tissue dysfunction.

Alternatively, fluctuations in morphogen levels may be assessed by monitoring morphogen transcription levels, either by standard Northern blot analysis as described in Example 1, or by in situ hybridization, using a  
5 labelled probe capable of hybridizing specifically to morphogen mRNA, and standard RNA hybridization protocols well described in the art and described generally in Example 1.

10 OP-1 was detected in human serum using the following assay. A monoclonal antibody raised against mammalian, recombinantly produced OP-1 using standard immunology techniques well described in the art and described generally in Example 15, was immobilized by  
15 passing the antibody over an agarose-activated gel (e.g., Affi-Gel<sup>TM</sup>, from Bio-Rad Laboratories, Richmond, CA, prepared following manufacturer's instructions) and used to purify OP-1 from serum. Human serum then was passed over the column and eluted with 3M  
20 K-thiocyanate. K-thiocyanate fractions then were dialyzed in 6M urea, 20mM PO<sub>4</sub>, pH 7.0, applied to a C8 HPLC column, and eluted with a 20 minute, 25-50% acetonitrile/0.1% TFA gradient. Since mature, recombinantly produced OP-1 homodimers elute between  
25 20-22 minutes, these fractions then were collected and tested for the presence of OP-1 by standard immunoblot using an OP-1 specific antibody as for Example 2.A.

Administered or endogenous morphogen levels may be  
30 monitored in the therapies described herein by comparing the quantity of morphogen present in a body fluid sample with a predetermined reference value, for example, to evaluate the efficiency of a therapeutic protocol, and the like. In addition, fluctuations in  
35 the level of endogenous morphogen antibodies may be

detected by this method, most likely in serum, using an antibody or other binding protein capable of interacting specifically with the endogenous morphogen antibody. Detected fluctuations in the levels of the morphogen or endogenous antibody may be used, for example, as indicators of a change in tissue status. For example, as damaged tissue is regenerated and the tissue or organ's function returns to "normal" and, in the absence of additional tissue damage, lower doses of morphogen may be required, and a higher level of circulating morphogen antibody may be measured.

Example 3. Morphogen Treatment of Oral Mucositis

Oral mucositis involves ulcerations of the mouth as a consequence of, e.g., radiation therapy or chemotherapy. The course of ulcerative mucositis may be divided into a destructive phase and a healing phase. Since the cells of the basal layer of the oral epithelium divide at a rapid rate, they are susceptible to the antimitogenic and toxic effects of chemotherapy. As a result, atrophic changes occur which then are followed by ulceration. This constitutes the destructive phase. Following ulcer formation, the lesions slowly resolve during the healing phase.

The example below demonstrates morphogen efficacy in protecting the oral mucosa from oral mucositis in a hamster model, including both inhibiting ulceration and enhancing regeneration of ulcerated tissue. Details of the protocol can be found in Sonis, et al., (1990) Oral Surg. Oral Med. Oral Pathol 69: 437-443, the disclosure of which is incorporated herein by reference. Briefly, golden syrian hamsters (6-8 wks old, Charles River Laboratories, Wilmington, MA) were divided into 3 test



groups: Group 1, a placebo (e.g., saline) control, and a morphogen low dose group (100 ng) and a morphogen high dose group (1  $\mu$ g), Groups 2 and 3, respectively. Morphogen dosages were provided in 30% ethanol. Each  
5 group contained 12 animals.

Beginning on day 0 and continuing through day 5, Groups 2 and 3 received twice daily morphogen applications. On day 3, all groups began the  
10 mucositis-induction procedure. 5-fluorouracil was injected intraperitoneally on days 3 (60 mg/kg) and 5 (40 mg/kg). On day 7, the right buccal pouch mucosa was superficially irritated with a calibrated 18 gauge needle. In untreated animals, severe ulcerative  
15 mucositis was induced in at least 80% of the animals by day 10.

For each administration of the vehicle control (placebo) or morphogen, administration was performed by  
20 first gently drying the cheek pouch mucosa, then providing an even application over the mucosal surface of the vehicle or morphogen material. A hydroxypropylcellulose-based coating was used to maintain contact of the morphogen with the mucosa.  
25 This coating provided at least 4 hours of contact time.

On day 12, two animals in each group were sacrificed for histological studies. The right buccal pouch mucosa and underlying connective tissue were  
30 dissected and fixed in 10% formalin using standard dissection and histology procedures. The specimens were mounted in paraffin and prepared for histologic examination. Sections then were stained with hematoxylin and eosin and were examined blindly by  
35 three oral pathologists with expertise in hamster

histology and scored blind against a standard mucositis panel. The extent of atrophy, cellular infiltration, connective tissue breakdown, degree of ulceration and epithelialization were assessed.

5

The mean mucositis score for each group was determined daily for each experimental group for a period of 21 days by photography and visual examination of the right buccal cheek pouch. Differences between groups were determined using the Students' 't' test. In addition, data was evaluated between groups by comparing the numbers of animals with severe mucositis using Chi Square statistical analysis. The significance of differences in mean daily weights also was determined.

The experimental results are presented in Figs. 1 and 2. Figure 1 graphs the effect of morphogen (high dose, squares; low dose, diamonds) and placebo (circles) on mean mucositis scores. Both low and high morphogen doses inhibit lesion formation significantly in a dose-dependent manner. Fig. 2 (A and B) are photomicrographs of a buccal cheek pouch on day 14, pretreated with morphogen, high dose (B) or saline alone (A). Significant tissue necrosis, indicated by the dark regions in the tissue, and ulceration, indicated by the light globular areas in the tissue, is evident in the untreated pouch in Fig. 2A. By contrast, the morphogen-treated tissue in Fig. 2B shows healthy tissue with no necrosis and little or no ulceration. In addition, histology results

consistently showed significantly reduced amounts of tissue atrophy, cellular debris, and immune effector cells, including activated macrophages and neutrophils, in the morphogen-treated animals, as compared with the untreated, control animals.

In a variation on this protocol, morphogen also may be administered daily for several days before mucositis-induction and/or for longer periods following 5-fluorouracil treatments.

Example 4. Morphogen Treatment of Duodenal Ulcer Formation

The following example provides a rat model for demonstrating morphogen efficacy in treating duodenal ulcers. A detailed description of the protocol is provided in Pilan et al., (1985) Digestive Diseases and Sciences 30: 240-246, the disclosure of which is incorporated herein by reference.

Briefly, Sprague-Dawley female rats (e.g., Charles River Laboratories, 150-200 grams) receive the duodenal ulcerogen cysteamine-HCl at a dose of 25-28 milligrams (mg) per 100 grams (gm) of body weight orally by intragastric gavage 3 times on the same day. Additionally, cortisol is administered subcutaneously to each rat at a single dose of 5mg of cortisol to 100 gm of body weight to decrease the mortality resulting from the administration of the cysteamine-HCl.

Three days after administration of the cysteamine-HCl, rats having penetrating and perforating duodenal ulcers are identified by standard laparotomy and randomized into control and morphogen-treated groups.

5

The rats of Group 1, all of which have ulcers, receive no morphogen and are treated only with saline. The rats of Group 2 each of which also have ulcers, receive 50-100 ng of morphogen per 100 gm of body weight. Group 3 rats, all of which have ulcers, receive 200-500 ng of morphogen per 100 gm of body weight. All treatments are by gavage twice daily until autopsy on day 21, when the ulcers are measured and histologic sections taken.

15

Histology of duodenal sections from morphogen-treated animals shows healed ulcers with prominent and dense granulation tissue and partial or complete re-epithelialization, demonstrating that oral administration of morphogen can significantly accelerate the healing of ulcers of the GI tract. Moreover, treatment with morphogen before or concomitantly with ulceration also inhibits ulcer formation.

25 Example 5. Gastric acid and Pepsin Secretion of Morphogen-Treated Rats

The following example demonstrates morphogen efficacy as determined by gastric acid and pepsin secretion. A detailed description of the protocol is provided in Pilan et al., disclosed above. Briefly, 18-20 rats are divided into 2 groups, a control group (Group 1) and a morphogen treated group (Group 2).

All rats are fasted for 24 hours and given either saline vehicle alone (Group 1) or morphogen (e.g., 500 ng/ml, Group 2). The stomachs of the rats then are constricted with a pyloric ligature for one hour.

5

Gastric juice is collected from each rat in groups 1 and 2, centrifuged and aliquots processed for acid titration to calculate gastric acid output and pepsin determination. Gastric acid is measured by the acidity of the gastric juices, and pepsin levels are determined according to standard protease assays well-known in the art. Since pepsin is the most abundant protease in the stomach, the total protease level is a good measurement of the pepsin level. The gastric juice aliquots are spectrophotometrically analyzed using albumin as a substrate. (Szabo, S. et al. (1977) Res. Comm. Chem. Pathol. Pharmacol. 16: 311-323, hereby incorporated by reference).

20 In both control and morphogen-treated rats normal levels of gastric pepsin output and gastric juice volume can be measured. Thus, morphogen treatment of ulcers of the GI tract does not affect the normal levels of gastric acid or pepsin in the GI tract.

25

Example 6. Morphogen Treatment of Ulcerative colitis

Ulcerative colitis involves ulcers of the colon. The example provided below demonstrates morphogen efficacy in treating ulcerative colitis using a guinea pig model. A detailed description of the protocol is provided in Onderdonk et al. (1979) Amer. J. Clin. Nutr. 32: 258-265, the disclosure of which is incorporated herein by reference.

35

Briefly, guinea pigs, (e.g., 500-550 gms, Charles River Laboratories) are divided into 3 experimental groups, each group containing multiple animals: a control, Group 1, which receives distilled water to drink; Group 2, which receives distilled water containing 1% degraded carrageenin; and Group 3, which receives distilled water containing 5% degraded carrageenin to drink. Degraded carrageenin is a polysaccharide derived from red seaweeds, (Glaxo Laboratories, Paris, France), and is a known inducer of ulcerative colitis in guinea pigs.

The development of colitis is determined using several criteria: 1) presence of loose and/or bloody feces by visual inspection, 2) detection of occult blood in the feces using Coloscreen III with hemocult developer (Helena Labs, Bumont, TX), and 3) weight loss.

At day 25, each animal is anesthetized with Ketamine (3-5 mg/kg) administered intramuscularly and a 3 mm colorectal mucosa biopsy taken using a small nasal scope. All of the specimens are fixed in 15% formaldehyde and examined histologically using hematoxylin and eosin. The pathologic diagnosis of ulcerative colitis is established by the presence of crypt abscesses, lymphocytic infiltration, capillary congestion of the lamina propria and ulceration of the colon mucosa (Onderdonk, (1985) Digestive Disease Science 30:40(s), hereby incorporated by reference). The severity of ulcerative colitis is graded on a scale of 0 to 3 and expressed as the pathological index according to the standard scoring system (Onderdonk et al. (1979), Amer. J. Clin. Nutrition 32:258.)

At day 30, 25% of the guinea pigs in which ulcerative colitis was demonstrated histologically are treated with morphogen and the remaining 25% receive distilled water as a control. Morphogen is administered both at a low dose (e.g., 100 ng/100 gm) in one half of the guinea pigs; and at a high dose (e.g., 500-1000 ng/100 gm), administered orally through a 3 mm bulbed needle, twice per day for a period of 10 days (days 28-37).

During treatment, the animals are evaluated clinically and improvements in body weight, stool consistency and reduction or absence of blood in stools recorded. At day 37, all animals are sacrificed with an overdose of pentobarbital (>200 mg/kg) and the entire colon removed for histological evaluation. Colon ulcers in morphogen treated animals are significantly repaired and healed as compared with untreated ulcers.

Example 7. Morphogen Inhibition of Epithelial Cell Proliferation

This example demonstrates the ability of morphogens to inhibit epithelial cell proliferation in vitro, as determined by <sup>3</sup>H-thymidine uptake using culture cells from a mink lung epithelial cell line (ATCC No. CCL 64, Rockville, MD), and standard mammalian cell culturing procedures. Briefly, cells were grown to confluency in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 200 units/ml penicillin, and 200 µg/ml streptomycin, and used to seed a 48-well cell culture plate at a cell density of 200,000 cells per well. When this culture became confluent, the media was replaced with 0.5 ml of EMEM

containing 1% FBS and penicillin/streptomycin and the culture incubated for 24 hours at 37 C. Morphogen test samples in EMEM containing 5% FBS then were added to the wells, and the cells incubated for another 18 hours. After incubation, 1.0  $\mu$ Ci of  $^3$ H-thymidine in 10  $\mu$ l was added to each well, and the cells incubated for four hours at 37 C. The media then was removed and the cells washed once with ice-cold phosphate-buffer saline and DNA precipitated by adding 0.5 ml of 10% TCA to each well and incubating at room temperature of 15 minutes. The cells then were washed three times with ice-cold distilled water, lysed with 0.5 ml 0.4 M NaOH, and the lysate from each well then transferred to a scintillation vial and the radioactivity recorded using a scintillation counter (Smith-Kline Beckman).

The results are presented in Fig. 3A and 3B. The anti-proliferative effect of the various morphogens tested was expressed as the counts of  $^3$ H-thymidine (x 1000) integrated into DNA. In this example, the biosynthetic constructs COP-5 and COP-7 were tested in duplicate: COP-7-1 (10 ng) and COP-7-2 (3 ng, Fig. 3A), and COP-5-1 (66 ng) and COP-5-2 (164 ng, Fig. 3B.) Morphogens were compared with untreated cells (negative control) and TGF- $\beta$  (1 ng), a local-acting factor also known to inhibit epithelial cell proliferation. COP-5 and COP-7 previously have been shown to have osteogenic activity, capable of inducing the complete cascade resulting in endochondral bone formation in a standard rat bone assay (see U.S. Pat. No. 5,011,691.) As is evident in the figure, the morphogens significantly inhibit cell epithelial cell proliferation. Similar experiments, performed with the morphogens COP-16 and bOP (bone-purified osteogenic protein, a dimeric protein comprising CBMP2 and OP-1) and recombinant OP-1



also inhibit cell proliferation. bOP and COP-16 also induce endochondral bone formation (see US Pat. No. 4,968,590 and 5,011,691.)

5 Example 8. Morphogen Inhibition of Cellular and Humoral Inflammatory Response

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10 Morphogens described herein inhibit multinucleation of mononuclear phagocytic cells under conditions where these cells normally would be activated, e.g., in response to a tissue injury or the presence of a foreign substance. For example, and as described in USSN [CRP059CP] in the absence of morphogen, an implanted substrate material (e.g., implanted  
15 subcutaneously) composed of, for example, mineralized bone, a ceramic such as titanium oxide or any other substrate that provokes multinucleated giant cell formation, rapidly becomes surrounded by multinucleated giant cells, e.g., activated phagocytes stimulated to  
20 respond and destroy the foreign object. In the presence of morphogen however, the recruited cells remain in their mononuclear precursor form and the matrix material is undisturbed. Accordingly, the morphogens' effect in maintaining the integrity of the  
25 GI tract luminal lining also may include inhibiting activation of these immune effector cells.

In addition, the morphogens described herein also suppress antibody production stimulated in response to  
30 a foreign antigen in a mammal. Specifically, when bovine bone collagen matrix alone was implanted in a bony site in a rat, a standard antibody response to the collagen was stimulated in the rat as determined by standard anti-bovine collagen ELISA experiments  
35 performed on blood samples taken at four week intervals

following implantation (e.g., between 12 and 20 weeks.) Serum anti-collagen antibody titers, measured by ELISA essentially following the procedure described by Nagler-Anderson et al, (1986) PNAS 83:7443-7446, the disclosure of which is incorporated herein by reference, increased consistently throughout the experiment. However, when the matrix was implanted together with a morphogen (e.g., OP-1, dispersed in the matrix and adsorbed thereto, essentially as described in U.S. Pat. No. 4,968,590) anti-bovine collagen antibody production was suppressed significantly. This ability of morphogen to suppress the humoral response is further evidence of morphogen utility in alleviating tissue damage associated with GI tract ulceration.

Example 9. Morphogen Effect on Fibrogenesis and Scar Tissue Formation

The morphogens described herein induce tissue morphogenesis of damaged or lost tissue. The ability of these proteins to regenerate new tissue enhances the anti-inflammatory effect of these proteins. Provided below are a series of in vitro experiments demonstrating the ability of morphogens to induce migration and accumulation of mesenchymal cells. In addition, the experiments demonstrate that morphogens, unlike TGF- $\beta$ , do not stimulate fibrogenesis or scar tissue formation. Specifically, morphogens do not stimulate production of collagen, hyaluronic acid (HA) or metalloproteinases in primary fibroblasts, all of which are associated with fibrogenesis or scar tissue formation. By contrast, TGF- $\beta$ , a known inducer of fibrosis, but not of tissue morphogenesis as defined herein, does stimulate production of these markers of fibrosis.

Chemotaxis and migration of mesenchymal progenitor cells were measured in modified Boyden chambers essentially as described by Fava, R.A. et al (1991) J. Exp. Med. 173: 1121-1132, the disclosure of which is incorporated herein by reference, using polycarbonate filters of 2, 3 and 8 micron pores to measure migration of progenitor neutrophils, monocytes and fibroblasts. Chemotaxis was measured over a range of morphogen concentrations, e.g.,  $10^{-20}$ M to  $10^{-12}$ M OP-1. For progenitor neutrophils and monocytes,  $10^{-18}$ - $10^{-17}$ M OP-1 consistently induced maximal migration, and  $10^{-14}$  to  $10^{-13}$ M OP-1 maximally induced migration of progenitor fibroblasts. In all cases the chemotactic activity could be inhibited with anti-OP-1 antibody. Similar migration activities also were measured and observed with TGF- $\beta$ .

The effect of morphogen on fibrogenesis was determined by evaluating fibroblast production of hyaluronic acid (HA), collagen, collagenase and tissue inhibitor of metalloproteinases (TIMP).

Human fibroblasts were established from explants of infant foreskins and maintained in monolayer culture using standard culturing procedures. (See, for example, (1976) J. Exp. Med. 144: 1188-1203.) Briefly, fibroblasts were grown in maintenance medium consisting of Eagle's MEM, supplemented with nonessential amino acids, ascorbic acid (50  $\mu$ g/ml),  $\text{NaHCO}_3$  and HEPES buffers (pH 7.2), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), amphotericin B (1  $\mu$ g/ml) and 9% heat inactivated FCS. Fibroblasts used as target cells to measure chemotaxis were maintained in 150 mm diameter glass petri dishes. Fibroblasts used in assays to measure synthesis of collagen, hyaluronic acid,

collagenase and tissue inhibitors of metalloproteinases (TIMP) were grown in 100 mm diameter plastic tissue culture petri dishes.

5       The effects of morphogen on fibroblast production  
of hyaluronic acid, collagens, collagenase and TIMP  
were determined by standard assays (See, for example,  
Posttethwaite et al. (1989) J. Clin. Invest. 83: 629-  
636, Posttethwaite (1988) J. Cell Biol. 106: 311-318  
10 and Clark et al (1985) Arch. Bio-chem Biophys. 241: 36-  
44, the disclosures of which are incorporated by  
reference.) For these assays, fibroblasts were  
transferred to 24-well tissue culture plates at a  
density of  $8 \times 10^4$  cells per well. Fibroblasts were  
15 grown confluency in maintenance medium containing 9%  
FCS for 72 h and then grown in serum-free maintenance  
medium for 24 h. Medium was then removed from each  
well and various concentrations of OP-1 (recombinantly  
produced mature or soluble form) or TGF- $\beta$ -1 (R&D  
20 Systems, Minneapolis) in 50  $\mu$ l PBS were added to  
triplicate wells containing the confluent fibroblast  
monolayers. For experiments that measured production  
of collagenase and TIMP, maintenance medium (450  $\mu$ l)  
containing 5% FCS was added to each well, and culture  
25 supernatants were harvested from each well 48 h later  
and stored at  $-70^\circ\text{C}$  until assayed. For experiments  
that assessed HA production, maintenance medium (450  
 $\mu$ l) containing 2.5% FCS was added to each well, and  
cultures grown for 48 h. For experiments that measured  
30 fibroblast production of collagens, serum-free  
maintenance medium (450  $\mu$ l) without non-essential amino  
acids was added to each well and cultures grown for 72  
h. Fibroblast production of HA was measured by  
labeling newly synthesized glycosaminoglycans (GAG)  
35 with [ $^3\text{H}$ ]-acetate the last 24 h of culture and

quantitating released radioactivity after incubation with hyaluronidase from Streptomyces hyalurolyticus (ICN Biochemicals, Cleveland, OH) which specifically degrades hyaluronic acid. Production of total collagen by fibroblasts was measured using a collagenase-sensitive protein assay that reflects [<sup>3</sup>H]-proline incorporation the last 24 h of culture into newly synthesized collagens. Collagenase and TIMP protein levels in fibroblast cultures supernatants was measured by specific ELISAs.

As shown in Fig. 4, OP1 does not stimulate significant collagen or HA production, as compared with TGF- $\beta$ . In the figure, panel A shows OP-1 effect on collagen production, panel B shows TGF- $\beta$  effect on collagen production, and panels C and D show OP-1 (panel C) and TGF- $\beta$  (panel D) effect on HA production. The morphogen results were the same whether the soluble or mature form of OP1 was used. By contrast, the latent form of TGF- $\beta$  (e.g., pro domain-associated form of TGF- $\beta$ ) was not active.

Example 10. Screening Assay for Candidate Compounds which Alter Endogenous Morphogen Levels

Candidate compound(s) which may be administered to affect the level of a given morphogen may be found using the following screening assay, in which the level of morphogen production by a cell type which produces measurable levels of the morphogen is determined with and without incubating the cell in culture with the compound, in order to assess the effects of the compound on the cell. This can be accomplished by detection of the morphogen either at the protein or RNA level. A more detailed description also may be found in USSN 752,861, incorporated hereinabove by reference.

## 10.1 Growth of Cells in Culture

Cell cultures of kidney, adrenals, urinary bladder,  
5 brain, or other organs, may be prepared as described  
widely in the literature. For example, kidneys may be  
explanted from neonatal or new born or young or adult  
rodents (mouse or rat) and used in organ culture as  
whole or sliced (1-4 mm) tissues. Primary tissue  
10 cultures and established cell lines, also derived from  
kidney, adrenals, urinary, bladder, brain, mammary, or  
other tissues may be established in multiwell plates (6  
well or 24 well) according to conventional cell culture  
techniques, and are cultured in the absence or presence  
15 of serum for a period of time (1-7 days). Cells may be  
cultured, for example, in Dulbecco's Modified Eagle  
medium (Gibco, Long Island, NY) containing serum (e.g.,  
fetal calf serum at 1%-10%, Gibco) or in serum-deprived  
medium, as desired, or in defined medium (e.g.,  
20 containing insulin, transferrin, glucose, albumin, or  
other growth factors).

Samples for testing the level of morphogen  
production includes culture supernatants or cell  
25 lysates, collected periodically and evaluated for OP-1  
production by immunoblot analysis (Sambrook et al.,  
eds., 1989, Molecular Cloning, Cold Spring Harbor  
Press, Cold Spring Harbor, NY), or a portion of the  
cell culture itself, collected periodically and used to  
30 prepare polyA<sup>+</sup> RNA for RNA analysis. To monitor de  
novo OP-1 synthesis, some cultures are labeled  
according to conventional procedures with an  
<sup>35</sup>S-methionine/<sup>35</sup>S-cysteine mixture for 6-24 hours and  
then evaluated to OP-1 synthesis by conventional  
35 immunoprecipitation methods.

## 10.2 Determination of Level of Morphogenic Protein

In order to quantitate the production of a morphogenic protein by a cell type, an immunoassay may be performed to detect the morphogen using a polyclonal or monoclonal antibody specific for that protein. For example, OP-1 may be detected using a polyclonal antibody specific for OP-1 in an ELISA, as follows.

10        1  $\mu$ g/100  $\mu$ l of affinity-purified polyclonal rabbit  
IgG specific for OP-1 is added to each well of a  
96-well plate and incubated at 37°C for an hour. The  
wells are washed four times with 0.167M sodium borate  
buffer with 0.15 M NaCl (BSB), pH 8.2, containing 0.1%  
15 Tween 20. To minimize non-specific binding, the wells  
are blocked by filling completely with 1% bovine serum  
albumin (BSA) in BSB and incubating for 1 hour at 37°C.  
The wells are then washed four times with BSB  
containing 0.1% Tween 20. A 100  $\mu$ l aliquot of an  
20 appropriate dilution of each of the test samples of  
cell culture supernatant is added to each well in  
triplicate and incubated at 37°C for 30 min. After  
incubation, 100  $\mu$ l biotinylated rabbit anti-OP-1 serum  
(stock solution is about 1 mg/ml and diluted 1:400 in  
25 BSB containing 1% BSA before use) is added to each well  
and incubated at 37°C for 30 min. The wells are then  
washed four times with BSB containing 0.1% Tween 20.  
100  $\mu$ l strepavidin-alkaline phosphatase (Southern  
Biotechnology Associates, Inc. Birmingham, Alabama,  
30 diluted 1:2000 in BSB containing 0.1% Tween 20 before  
use) is added to each well and incubated at 37°C for 30  
min. The plates are washed four times with 0.5M Tris  
buffered Saline (TBS), pH 7.2. 50 $\mu$ l substrate (ELISA  
Amplification System Kit, Life Technologies, Inc.,  
35 Bethesda, MD) is added to each well incubated at room

temperature for 15 min. Then, 50  $\mu$ l amplifier (from the same amplification system kit) is added and incubated for another 15 min at room temperature. The reaction is stopped by the addition of 50  $\mu$ l 0.3 M sulphuric acid. The OD at 490 nm of the solution in each well is recorded. To quantitate OP-1 in culture media, a OP-1 standard curve is performed in parallel with the test samples.

10 Polyclonal antibody may be prepared as follows. Each rabbit is given a primary immunization of 100  $\mu$ g/500  $\mu$ l E. coli produced OP-1 monomer (amino acids 328-431 in SEQ ID NO:5) in 0.1% SDS mixed with 500  $\mu$ l Complete Freund's Adjuvant. The antigen is injected subcutaneously at multiple sites on the back and flanks of the animal. The rabbit is boosted after a month in the same manner using incomplete Freund's Adjuvant. Test bleeds are taken from the ear vein seven days later. Additional boosts and test bleeds are performed at monthly intervals until antibody against OP-1 is detected in the serum using an ELISA assay. Then, the rabbit is boosted with 100  $\mu$ g of antigen and bled (15 ml per bleed) at days seven and ten after boosting.

25 Monoclonal antibody specific for a given morphogen may be prepared as follows. A mouse is given two injections of E. coli produced OP-1 monomer. The first injection contains 100 $\mu$ g of OP-1 in complete Freund's adjuvant and is given subcutaneously. The second injection contains 50  $\mu$ g of OP-1 in incomplete adjuvant and is given intraperitoneally. The mouse then receives a total of 230  $\mu$ g of OP-1 (amino acids 307-431 in SEQ ID NO:5) in four intraperitoneal injections at various times over an eight month period. One week prior to fusion, the mouse is boosted intraperitoneally



with 100  $\mu$ g of OP-1 (307-431) and 30  $\mu$ g of the N-terminal peptide (Ser<sub>293</sub>-Asn<sub>309</sub>-Cys) conjugated through the added cysteine to bovine serum albumin with SMCC crosslinking agent. This boost was repeated five days  
5 (IP), four days (IP), three days (IP) and one day (IV) prior to fusion. The mouse spleen cells are then fused to myeloma (e.g., 653) cells at a ratio of 1:1 using PEG 1500 (Boeringer Mannheim), and the cell fusion is plated and screened for OP-1-specific antibodies using  
10 OP-1 (307-431) as antigen. The cell fusion and monoclonal screening then are according to standard procedures well described in standard texts widely available in the art.

15 The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the  
20 invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

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